To Peter

with thanks

Greg

P. D. Cooper.
JCSMR
Oct. 1969.

Chemical and Genetic Studies with Poliovirus

by

Gregory Austin Tannock

A thesis submitted for the degree of
Doctor of Philosophy in the Australian
National University

Department of Microbiology,
The John Curtin School of Medical Research.

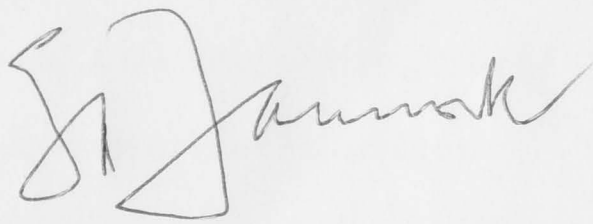
October 1969.

STATEMENT

The experimental work reported in this thesis was carried out entirely by myself, but some aspects of the work involved the use of material supplied by others. This included:

1. Temperature-sensitive mutants of poliovirus obtained by use of the mutagen 5-fluorouracil and used extensively in Experimental Section 2. These mutants were isolated and characterised by Dr P.D. Cooper in collaboration with Drs B.B. Wentworth and D. McCahon.

2. Certain radioactively labelled viral markers, which were obtained from Drs A.J. Gibbs and C.J. Burrell, as indicated in Materials and Methods.



SUMMARY

The Introduction comprises (A) a review of principal events that occur during the replication of poliovirus (B) a consideration of poliovirus genetics, paying particular attention to the use of temperature-sensitive mutants and (C) a brief outline of methods currently available for determining the molecular weight of viral RNA.

The experimental work is reported in three sections. The first describes the isolation of ts mutants of poliovirus after treatment of poliovirus infectious RNA with the mutagen nitrous acid or the exonuclease snake venom phosphodiesterase. The kinetics of inactivation for either reagent was studied. It was hoped to produce deletion mutants at the 3′-terminus of the viral RNA with venom phosphodiesterase.

Three of the best of the mutants were used in recombination tests and each was shown to have at least one defect in the region of the genome concerned with viral RNA synthesis. Reasons are given for believing that the mutants obtained after treatment with snake venom phosphodiesterase did not arise from exonuclease activity.

In the Second Section, a polymerase assay for poliovirus strain ts^{+} in U cells is described, and its principal characteristics are outlined. Maximum enzyme

levels were obtained in cells after 6 hours of ts$^+$ infection at 37.2^o C, and the RNA product consisted of a complex of viral single- and double- stranded material. The double- stranded RNA of the complex had a similar sedimentation coefficient and thermal transition properties to that of *in vivo* labelled Replicative Form RNA.

All ts mutants and ts$^+$ induced lower levels of polymerase after growth in cells at 39.5^o C. The optimum temperature for the induction of ts-20 polymerase was shown to be 34.5 - 35^o C compared with 37^o C for ts$^+$. *In vitro* studies indicated that the ts defect did not result from thermolability caused by alterations to the completed mutant polymerase molecule due to amino acid substitution.

In Experimental Section 3 attempts were made to compare the molecular weight of poliovirus RNA with several well characterised RNA molecules by (a) sucrose-gradient centrifugation under conditions in which RNA secondary structure was minimised and, (b) electrophoresis in polyacrylamide gels.

In sucrose-formaldehyde gradients poliovirus RNA appeared to have a sedimentation coefficient slightly higher than that of TMV RNA, suggesting that the molecular

weight of poliovirus RNA was in the range 2.1 - 2.2 x 10^6 daltons. However, this figure is open to question in consequence of a similar comparative study using poliovirus and bacteriophage R17 RNA; relatively poor resolution was achieved in the presence of formaldehyde, despite an anticipated two fold difference in their molecular weights. The contribution of residual secondary structure remains unknown. Semliki Forest virus (SFV) RNA, as represented by its 26 S 'interjacent' form, appears to have a molecular weight of between 1 and 2 x 10^6 daltons.

Much better resolution was achieved between various RNA species after electrophoresis in 2.4 per cent polyacrylamide gels. The molecular weight of poliovirus RNA, obtained by extrapolation in comparison with other markers, was shown to be 2.4 - 2.65 x 10^6 daltons. The effect of RNA secondary structure on such an estimation is also unknown.

Findings from the three Experimental Sections are finally discussed in relation to one another and as a basis for further studies.

INTRODUCTION

Poliovirus is a small icosahedral virus of diameter about 30 mμ whose complete particle or virion comprises a single length of ribonucleic acid (RNA) (Schaffer, 1962) which is surrounded by a protein capsid containing four or five proteins (Summers, Maizel and Darnell, 1965; Cooper, 1969). The host range of the virus is restricted to certain primate cells and specificity is determined by lipoprotein receptor substances in the cell membrane (Holland and Hoyer, 1962).

Poliovirus is probably the best studied of all animal viruses and the reasons for this are, in part, historical - poliovirus being the identifiable agent of a serious human affliction - and, in part, due to certain unique properties of the virus. Poliovirus is at once highly stable, easily cultivable and assayed in cell culture and readily purified. It also possesses the advantage of having a relatively small genome (molecular weight about 2×10^6 daltons), which implies a mechanism of replication that is simple in comparison with other animal viruses. Finally, replication occurs in the absence of normal cellular DNA function, which considerably simplifies the biochemical elucidation of the infectious process.

The introduction to this thesis comprises a review of several different aspects of recent work related to the

Experimental Sections. It commences with a description of the principal events in the infectious cycle of poliovirus and is followed by a consideration of its genetics. Finally, a short review will be given of methods currently available for the estimation of the molecular weight of viral RNA.

A. Principal Features of the Growth of Poliovirus

A.1 Characteristics of the Growth Cycle

The growth cycle of poliovirus in cultured cells has been investigated by a number of workers (Howes and Melnick, 1957; Darnell, Levintow, Thoren and Hooper, 1961 Cooper, 1964 a). For poliovirus type I growing in ERK cells the latent period, defined operatively as the earliest time at which one infectious unit per cell is produced, has been shown to be about 2.6-2.8 hours. This time is characterised by random delays within individual cells associated with the uptake or eclipse of the infecting virus, but the probability of such a delay can be greatly lessened by the use of higher multiplicities of infection (Cooper, 1964 a). Virus titres continue to increase after the onset of the latent period up to 6 hours after infection, when maximum titres are reached. Release of virus from individual cells is always preceded

by maturation (Howes, 1959) and takes place after a variable delay, often of the order of several hours.

A.2 Early Stages of Infection

A.2.1 Adsorption

The initial attachment of poliovirus to susceptible cells occurs between virion proteins and specific receptors on the cell surface. Adsorption is relatively insensitive to temperature, but is dependant upon the presence of specific cation concentrations, implying some kind of electrostatic interaction. It is completely reversed at low pH, by sodium deoxycholate or by very high salt concentrations (Philipson, 1963; Fenwick and Cooper, 1962). Attachment of poliovirus is specific for certain primate cells and cell fractionation studies indicate that specificity is associated with insoluble lipoproteins found in the microsomal fraction, which are associated with the cytoplasmic membrane (Holland and McLaren, 1961). Other primate cells to which poliovirus adsorbs relatively inefficiently *in vivo*, when cultured *in vitro* acquire the same capacity for adsorption as that of susceptible cells (Holland and Hoyer, 1962).

Probably the best demonstration of the specificity of virion protein/cell receptor interactions has been that of Cords and Holland (1964). By mixedly infecting HeLa

cells with poliovirus type I and coxsackie virus B1, they were able to produce high yields of 'mixed' virions, which included poliovirus RNA enclosed within coxsackie virus B1 virion proteins. Such virions were then shown to infect cultured mice fibroblasts or suckling mice and to produce single cycle yields of poliovirus, despite the fact that both hosts are normally refractory to infection by poliovirus.

The specificity of the virion protein/host receptor interaction may be by-passed by the use of infectious RNA (Darnell and Sawyer, 1960), which may also remove chance delays which occur at or near the cell surface at the initiation of infection (Cooper, 1964 b).

A.2.2 Penetration and uncoating

There is much evidence that, for poxviruses and myxoviruses, the transport of the infecting virus particle across the cell membrane occurs by a process of engulfment, whereby the particle becomes enclosed by processes of the membrane. Uncoating then occurs within the vacuoles. Such a mechanism is probably a normal cellular process and not one specifically adapted to transport of infecting particles. Among the most compelling evidence for this engulfment process, termed 'viropexis' by Fazekas de St. Groth (1948), are electron

micrographic studies (Silverstein and Marcus, 1964; Dales and Kajioka, 1964), which clearly show stages of the invagination process for Newcastle disease and vaccinia viruses and demonstrate the enclosure of the virions within vacuoles beneath the cell surface. With poliovirus the situation may be different and there is evidence that the actual uncoating process may occur at or near the cell membrane. The actual eclipse process for poliovirus can be divided into several stages in temperature dependance. If adsorption is allowed to take place at 0^{o} and the temperature then raised above $23^{o}C$, as much as 60-80 per cent of adsorbed virus is rapidly eluted (Joklik and Darnell, 1961; Fenwick and Cooper, 1962). The eluted virus contains normal amounts of infectious RNA, but the capsid proteins are somewhat altered in their physico-chemical properties. Attachment of the remaining fraction is followed first, by an acid-reversible reduction in infectivity by the cell and then by the acquisition of resistance to neutralising antibody (Fenwick and Cooper, 1962).

Mandel (1967) has presented evidence for an intracellular site for the uncoating of poliovirus. Using light sensitive virus prepared by growing in the presence of neutral red (Wilson and Cooper, 1962), he was able to

observe differences in the times and rates at which the transitions 1 to resistance to neutralisation, 2 to photoinactivation (at which he considered RNA release to occur) and 3 to final nonrecoverability were attained. After adsorption at 25°, Mandel observed that virus which 'penetrated' the infecting cells, (i.e. was resistant to antiserum) could be obtained as fully infectious photosensitive material upon cell lysis. Mandel, however, did not rule out a situation whereby the initiation of uncoating was extracellular, due to derangement of the virion proteins by the cell membrane and the final RNA release occurred within vacuoles similar to those demonstrated near the surfaces of cells infected with poxviruses or myxoviruses.

A.3 Molecular events which occur during replication

A.3.1 Site of virus replication

There is now much evidence that the entire replication of poliovirus takes place within the cytoplasm of infected cells. This is suggested by cell fractionation studies (Darnell, 1962; Holland and Bassett, 1964) and from studies of poliovirus antigen synthesis in anucleate cytoplasm (Crocker, Pfendt and Sprendlove, 1964; Marcus and Freeman, 1966). Replication has been shown to take place in the absence of cellular DNA function, as is

evidenced by its growth in the presence of actinomycin D (AMD), a potent inhibitor of cellular transcription (Reich, Franklin, Shatkin and Tatum, 1961). More recent reports, however, indicate that, under some conditions, the growth of poliovirus is actually inhibited by AMD (Grado, Fischer and Conteras, 1965; Cooper, 1966; Schaffer and Gordon, 1966). Cooper (1966) in a careful analysis of the situation in poliovirus infected U cells was able to induce specifically the condition of AMD sensitivity under conditions of cellular insulin starvation. The precise mechanism for this phenomenon is unknown, but it seems likely from this and other studies on the suppression of cellular RNA synthesis (see next section) that the physiological state of the cell is a determinant of events in the infectious process.

A.3.2 Effects on host cell macromolecular synthesis

A well characterised feature of the infectious process is the appearance after about three hours of polyribosomes with a much higher sedimentation coefficient than are present in uninfected cells (Penman, Scherrer, Becker and Darnell, 1963; Summers and Levintow, 1965). From electron micrographs, ribosomes of infected cells appear in clusters, unlike those from uninfected cells, which are dispersed throughout the cytoplasm (Penman et al., 1963). The

existence of lipoprotein vesicles surrounding the ribosome clusters is suggested by the need to treat cytoplasmic extracts of infected cells with surface active agents, such as sodium deoxycholate, in order to demonstrate polyribosomes by sedimentation analysis (Penman, Becker and Darnell, 1964). These supposed vesicles have been termed 'Virus Synthesising Bodies' and similar findings have been noted for cells infected with encephalomyocarditis virus (EMC) by Dalgarno and Martin, (1965).

The change in the polyribosome profile that occurs in response to infection may be mediated by a viral coded protein. This is suggested by the work of Willems and Penman (1966) who showed that a puromycin-sensitive step was necessary for polyribosome synthesis.

Inhibition of host cellular RNA and protein synthesis, and of ribosome maturation, has been described as a feature of infection by poliovirus (Salzman, Lockart and Sebring, 1959; Fenwick, 1963; Zimmerman, Heeter and Darnell, 1963; Holland 1963; Bablanian, Eggers and Tamm, 1965), although the actual time of commencement of inhibition so described varies from 1.5 to 3.0 hours after infection. Some earlier reports had suggested that lasting stimulation of cellular RNA synthesis actually occurred in response to infection (Maasab, Loh and Ackermann, 1957;

Ackerman, Loh and Payne, 1959), while in others (Conteras, Tohá and Ohlbaum, 1959; Levy, 1961) the stimulation of cellular RNA Synthesis appeared rather transitory in nature. That inhibition of cellular RNA synthesis, where it occurs, is at least partly dependant upon the nature and condition of the infected cell is suggested from the work of Plagemann and Swim (1966) using mengovirus, another picornavirus. They found that depression in host cell RNA and protein synthesis occurred in infected cells from one cell line but not in another derived from the first cell line.

Where inhibition of host cell RNA and protein synthesis occurs after infection, the rate of inhibition has been demonstrated to be faster than that induced by the addition of AMD to uninfected cells (Penman et al., 1963). This suggests that inhibition occurs by a more complex mechanism than the mere blocking of cellular DNA transcription. In virus infected cells treated with guanidine, the synthesis of viral RNA is arrested but inhibition of host cell RNA and protein goes on unimpeded (Bablanian et al., 1965). Such inhibition is blocked by puromycin, but not by actinomycin D, which indicates that a protein, coded for by RNA from the infecting virus, is involved (Penman and Summers, 1965).

Inhibition of cellular DNA synthesis occurs as a result of infection (Salzman et al., 1959; Holland and Peterson, 1964), although preformed cellular DNA is apparently not degraded. Inhibition first occurs 3 - 4 hours after infection, or somewhat later than the inhibition of cellular RNA and protein synthesis and is probably related to a decline in levels of DNA primed-RNA polymerase in infected cells reported by Holland (1962) and Holland and Peterson (1964). The genetic nature of this inhibition is suggested by Cooper, Johnson and Garwes (1966), who obtained mutants of poliovirus which were defective in their capacity to inhibit DNA synthesis in infected cells.

In summary, considerable changes in normal host cell macromolecular synthesis frequently occur as a result of infection and there is much evidence to support the view that such changes are manifest by novel protein(s), coded for by the RNA of the infecting virus. However the postulation of some kind of interplay between the host cell and the inhibitory process is probably necessary, in order to account for the many instances where inhibition has not been observed.

A.3.3 Viral RNA Replication

(a) Kinetics and location of RNA synthesis

The synthesis of poliovirus RNA has been demonstrated

to commence about 30-60 minutes after infection (Baltimore, Girard and Darnell, 1966) and continues exponentially during the first 2.5 hours before slowing down. Viral RNA synthesis demands the concomittant synthesis of protein and may be inhibited by the addition of antagonists of protein synthesis, such as puromycin and p-fluorophenylalanine (Wecker and Schonne, 1961; Levintow, Thoren, Darnell and Hooper, 1962).

The actual site for viral RNA replication appears to be the Poliovirus Replication Complex (Girard, Baltimore and Darnell, 1967). These are large bodies, heterogeneous in size, but with a mean sedimentation value of 250S, which can be released from Virus Synthesising Bodies with sodium deoxycholate. The Replication Complex contains no ribosomes and may be broken down with pronase or sodium dodecyl sulphate into a smaller structure sedimenting at 30-70S. Viral RNA polymerase activity is associated with the Replication Complex, as indeed are the main RNA intermediates in the replication of poliovirus RNA.

(b) Events common to the replication of bacteriophage and viral RNA

The replication of poliovirus RNA and that of other small RNA containing animal viruses has many features in common with the replication of RNA bacteriophages. For

both replication takes place in the absence of DNA function (Reich et al., 1961) and many RNA intermediates in the synthesis of viral RNA are similar (Bishop, Summers and Levintow, 1965; Erikson and Franklin, 1966). Much of recent insight into the replication of viral RNA derives from two findings with cells infected with animal viruses.

The first of these was the demonstration (Montagnier and Sanders, 1963) in Krebs ascites cells infected with encephalomyocarditis virus that, of the RNA species produced in response to infection, a small component (about 1-5 per cent of total RNA) is resistant to the action of ribonuclease and has many features of a double-stranded RNA polynucleotide of approximately twice the molecular weight of viral RNA. Formation of double stranded RNA is now recognised as a feature of the replication of many small RNA viruses including poliovirus (Weissmann, Borst, Burdon and Ochoa, 1964; Baltimore, Becker and Darnell, 1964; Nayak and Baluda, 1968).

The second finding was that of Baltimore and Franklin (1963) who were able to demonstrate an enzymic activity in the cytoplasm of mengovirus infected L-cells, which was absent in uninfected cells. This activity was responsible for the incorporation of ribonucleoside triphosphate into acid precipitable material with the properties of RNA, in

the presence of the other three ribonucleoside triphosphates. Such an enzyme has been variously referred to as an RNA polymerase, replicase, synthetase or nucleotidyl transferase and there is good evidence of its induction in response to infection by a wide range of animal, plant and bacterial RNA viruses (Weissmann, Simon and Ochoa, 1963; Polatnick and Arlinghaus 1967; Gilliland and Symons, 1968). An RNA polymerase activity in poliovirus infected cells was first described by Baltimore (1964) in HeLa cells infected with type II poliovirus. Baltimore characterised the *in vitro* RNA product of this enzyme and obtained a similar profile to that observed *in vivo* in poliovirus infected cells.

The polymerase of the RNA bacteriophage Qβ has been very highly purified and shown to exhibit considerable specificity for viral RNA in *in vitro* RNA synthesising systems (Spiegelman, Haruna, Holland, Beaudreau and Mills, 1965). Studies of the same kind have not been possible with animal virus polymerase preparations, which rapidly lose activity upon purification (Martin, 1967) and cannot be specifically primed by the addition of viral RNA (Girard, 1969).

(c) RNA produced in vivo by poliovirus

Three types of RNA have been identified in poliovirus infected cells which are not present in uninfected cells. Of these, the predominant species is single stranded 35S RNA which is indistinguishable from the RNA prepared from purified poliovirions (Darnell, 1962). A second form of RNA is the double stranded material referred to previously, which consists of one strand of viral RNA (the positive strand) and a complementary (negative) strand in a hydrogen bonded duplex structure (Baltimore, 1966). The duplex is referred to as the Replicative Form and has a sedimentation coefficient of 18-20S.

The third identifiable species is a complex of single and double stranded RNA which sediments heterogeneously at between 18 and 35S and is referred to as the Replicative Intermediate (Baltimore and Girard, 1966). The complex may be converted to the Replicative Form by the action of ribonuclease or by heating to between 60° and 75°C - well below the temperature of strand separation for the Replicative Form (Baltimore, 1968; Bishop and Koch, 1969).

(d) The Role of double stranded RNA

Similar RNA intermediates have been described for bacterial cells infected with RNA bacteriophages (Erikson

and Franklin, 1966), although some doubt has been cast on the reality of double stranded RNA in the *in vivo* situation by Weismann's group (Feix, Slor and Weissmann, 1967). They contend that double stranded viral RNA is an artefact produced by the deproteinisation procedure used in the preparation of RNA from infected cells. Using *in vitro* RNA synthesising systems and highly purified polymerase, they have demonstrated that complementary negative strands, alone, serve as the templates for viral replication (Feix, Pollet and Weissmann, 1968) and suggest that, *in vivo*, these occur in fairly loose association with viral RNA. Attempts to demonstrate *in vitro* synthesis of viral RNA using either Replicative Form or Replicative Intermediate as the template have been unsuccessful (Feix et al., 1968; Mills, Pace and Spiegelman, 1966). Whether the same situation obtains for animal virus polymerases is unknown, since it has not been possible to prepare polymerases free of viral RNA. Despite Weissmann's claim, however, double stranded RNA, prepared by phenol extraction of poliovirus infected cells, occurs as a highly ordered structure with respect to resistance to ribonuclease, sedimentation coefficient and thermal transition properties (Bishop and Koch, 1967). It is difficult to envisage the reproducible formation of so ordered a structure by an annealing mechanism.

(e) Models of RNA replication

There is general agreement that complementary strands of RNA are synthesised in response to infection by RNA viruses in an asymmetric manner, a considerable excess of positive over negative strands being produced (Erikson, Fenwick and Franklin, 1965; Bishop et al., 1965). Putting aside the objections of Weissmann, two principal models of RNA replication have been proposed, both of which assume the formation of a hydrogen bonded duplex structure as a necessary intermediate. In the first or conservative model, which operates in the replication of the single stranded DNA bacteriophage ϕ x 174 (Denhardt and Sinsheimer, 1965), the duplex remains intact throughout the time of replication and for the synthesis of progeny positive strands it is necessary to postulate some transient separation of the two strands, which would, however, still leave the duplex resistant to ribonuclease.

In the alternative or semi-conservative model first proposed by Weissmann et al., (1964), the positive strand of the original duplex is displaced by growing strands of RNA complementary to the negative strand. After displacement, in either model the positive strand becomes available to form new duplex structures, to serve as messengers in the synthesis of virion or non-virion proteins, or to become

incorporated as the RNA genome of completed virus particles.

According to the conservative model, labelled RNA from the infecting virus should enter the double stranded duplex and remain there during replication. Studies of this kind with animal viruses are, however, complicated by the very high particle to infectivity ratio, which for poliovirus may be as high as 1000 (Fenwick and Cooper, 1962). With the RNA bacteriophage MS2, Kelly and Sinsheimer (1967) were able to detect parental label in an RNA duplex structure soon after infection, but could find no evidence that such a structure was conserved during the period of replication.

Another prediction of the conservative hypothesis concerns the intactness of the positive strand of the RNA duplex obtained by ribonuclease digestion (Martin, 1966). According to the conservative model, when strands of the duplex are separated by heating the positive strand should be the same size as intact viral RNA. By the semi-conservative model, the positive strand would be fragmented, with each fragment representing the degree of base pairing between a nascent positive strand and the negative strand of the template. Experiments of this kind have been carried out with the Replicative Form of poliovirus RNA and these indicate that the positive strand is intact (Katz and

Penman, 1966). Similar findings had previously been made for the Replicative Form of R17 bacteriophage and foot-and-mouth disease virus (Erikson et al., 1965; Brown and Martin, 1965).

Further evidence for the intactness of the positive strand of the Replicative Form of poliovirus is its infectivity (Pons, 1964; Koch, Quintell and Bishop, 1966). During infection strand separation of the infecting duplex presumably takes place in order for the synthesis of viral 'early' proteins to occur, and it seems likely that an intact positive strand, rather than the negative strand, is required as messenger RNA. Two pieces of evidence support this contention. The first is the demonstration that single breaks in poliovirus RNA completely abolish its infectivity (Gierer, 1957; Ginoza, 1958) and the second is the demonstration that purified negative strands which are induced by RNA bacteriophage MS2 are non-infectious (Feix et al., 1968).

Some support for semi-conservative replication with poliovirus has been provided by Baltimore (1968), who had previously shown (Baltimore and Girard, 1966) that, by pulse labelling for 2.75 minutes, most label entered the RNA Replicative Intermediate. Such a situation is in contrast to the normal profile obtained when labelling was

carried out for longer periods as described above. Baltimore (1968) was able to convert pulse-labelled Replicative Intermediate into Replicative Form with ribonuclease or by gentle heating and found that a high proportion of radioactivity (about 37 per cent) was resistant to ribonuclease, implying that a long length of growing strand is hydrogen-bonded to the template. By hybridisation studies, Baltimore found that complementary RNA in the Replicative Intermediate was very rapidly synthesised, and these findings are consistent with a semi-conservative model. He calculated that there are on the average 6.5 growing strands per double stranded template.

There is evidence with the RNA bacteriophage MS2 that both conservative and semi-conservative forms of replication can occur simultaneously. Kelly and Sinsheimer (1967) have found that, when the nascent RNA of the Replicative Intermediate is labelled with a short pulse of radioactivity, only about half of the label is displaced from the Replicative Form. Similarly when the parental RNA is labelled and incorporated into a duplex structure, only about half of the label is displaced. On the basis of this they argue that an equal probability exists that the replication of the infecting RNA will occur by the conservative or the semi-conservative mode. One weakness of their case for

conservative replication, however, is their inability to detect the conservation of duplex structures throughout the replication cycle.

(f) Differences between RNA bacteriophage and animal virus induced polymerases

It is possible that many of the findings for RNA bacteriophage replication have no parallel in the replication of animal viruses, as it seems likely that greater complexity could be expected in the replication of animal viruses, with a genetic component almost twice as great as that of the RNA bacteriophages (See Experimental Section 3). One cause of this complexity may be the need for more than one virus-coded polymerase in the synthesis of the duplex and nascent positive strands in the case of animal viruses.

That two polymerase activities exist in cells infected with RNA bacteriophages, seems evident from studies by Lodish and Zinder (1966), who were able to isolate a mutant of RNA bacteriophage f2 which was defective in its capacity to synthesise double stranded RNA at restrictive temperatures. Studies with purified polymerase induced by RNA bacteriophage Qβ suggest that a single enzyme is involved, although certain host co-factors are simultaneously required for activity (Eikhom, Stockley and Spiegelman,

1968; Franze de Ferdinandez, Eoyang and August, 1968). These host factors may function as the second enzyme necessary for synthesis of nascent RNA, although their sequential addition to reaction mixtures has not specifically demonstrated this.

Genetic evidence has been presented by Cooper, Summers and Stancek (1969) for the existence of two separate polymerases which are coded for by poliovirus RNA. They examined the RNA profiles of two mutants, both of which had been previously shown to be defective in their capacity to synthesise viral RNA when grown under restrictive conditions. The defects of each mutant occurred fairly close together in what was appeared to be the polymerase gene of the poliovirus genome (Figure 1). One mutant (ts - 20) was seen to be defective in its capacity to induce the synthesis of both Replicative Form and viral RNA. The other mutant (ts - 28), whose defect was more close in the genetic map to the genes specifying virion proteins, was able to synthesise Replicative Form but was defective in its ability to synthesise viral RNA.

Undoubtedly the best evidence for the existence of two separate viral coded polymerase activities in picornavirus infected cells is that of Arlinghaus and Polatnick (1969) with the polymerase of foot-and-mouth disease virus.

They were able to fractionate a soluble RNA polymerase from membrane particulates of infected cells into two complexes. The lighter of the two complexes (20-70S) synthesised only 20S double stranded material and the heavier complex (100 - 300S) synthesised only 37S viral RNA. A third much larger complex which synthesised both 37S and double stranded RNA was considered to be the native polymerase complex still bound to cellular particulate material.

A.3.4 Messenger function of viral RNA

Direct evidence of messenger function for poliovirus RNA has been provided by Warner, Madden and Darnell (1963), using an *E. coli* protein synthesising system. They noted a marked uptake of labelled amino acids into large structures (about 100S) containing proteins which reacted with antisera prepared against guanidine-iodoacetamide treated suspensions of poliovirus. Similar experiments with HeLa cell extracts were unsuccessful, due possibly to inefficient interaction between ribosomes and viral messenger (Summers and Levintow, 1965). Failure here may also reflect an increase of complexity in the initiation of translation for mammalian cells compared with bacterial cells, which renders the conditions for *in vitro* protein synthesis more difficult to achieve. A similar lack of success has marked the efforts of many workers in

attempting to demonstrate messenger function for 9S RNA from rabbit reticulocytes, which possesses many of the properties of haemoglobin messenger (see review by Chantrenne, Burny and Marbaix, 1967).

Messenger function for RNA of bacteriophages has been demonstrated in cell free bacterial systems (Ohtaka and Spiegelman, 1963; Nathans, Oeschger, Eggen and Shimura, 1963; Viñuela, Algranati and Ochoa, 1967).

There are, however, other indications that protein synthesis specified by viral RNA takes place in infected cells. Scharff, Shatkin and Levintow (1963) pulse-labelled infected cells with radioactive amino acids and were able to isolate growing peptide chains from large polyribosomes, which are characteristic of poliovirus infected cells. About 25 per cent of the growing chains reacted with antisera prepared against poliovirus capsid protein. Penman et al. (1964) isolated the RNA from viral polyribosomes and found it to have a similar sedimentation coefficient to viral RNA and Summers and Levintow (1965) found similarities in their base compositions.

A.3.5 Translation by poliovirus RNA

Recent suggestions that fundamental differences exist in the translation mechanisms of bacterial and mammalian cells appear to have considerable implications for viral

protein synthesis in cells infected with RNA viruses. Many bacterial messenger RNAs and the RNA of certain bacteriophages are considered to be polycistronic, with protein chain initiation and termination being specified at intervals along the RNA messenger (Viñuela et al., 1967; Morse, Baker and Yanofsky, 1968). Some mammalian messengers, on the other hand, are probably monocistronic, a single RNA molecule specifying each polypeptide.

A growing body of evidence supports this contention and has been outlined by Jacobson and Baltimore (1968 a). Briefly, support comes from the observation that separate messengers within individual cells specify each polypeptide chain of haemoglobin (Warner, Knopf and Rich, 1963) and IgG gamma globulin (Shapiro, Scharff, Maizel and Uhr, 1966; Williamson and Askonas, 1967). Further corroboration comes from the observation of a linear relationship between the size of nascent polypeptides and the number of ribosomes attached to polyribosomes in mouse plasma tumour cells (Kuff and Roberts, 1967), HeLa cells (Baltimore and Huang, 1968), and chick embryo fibroblasts (Kretsinger, Manner, Gould and Rich, 1964). By contrast, the nascent polypeptide chains from bacterial messenger RNA appear to be the same length for all polyribosome sizes (Kiho and Rich, 1964; Hotham-Iglewski and Franklin, 1967).

Since the genome of poliovirus consists of a single length of RNA (Schaffer, 1962), the suggestion has been made that a monocistronic message is translated to produce a large protein, which is subsequently cleaved by specific proteases to smaller capsid and non-capsid proteins. This suggestion arises from the findings of Summers et al. (1965) who examined the proteins of infected cells by polyacrylamide gel electrophoresis. They obtained synchronous viral-specific protein synthesis in infected cells by blocking the infectious process with guanidine, which still allows viral induced inhibition of host cell protein synthesis, and then reversing the inhibition by removal of the drug. A pattern of fourteen polypeptides was obtained, of which four were identifiable as virion polypeptides. The combined molecular weights of capsid and non-capsid proteins was subsequently shown to approximate 530,000 (Summers and Maizel, 1968), a figure equivalent to a genetic information content of more than twice specified by a molecule of RNA of molecular weight 2×10^6, which is generally accepted for poliovirus (Cooper, 1969).

The only way of accounting for the discrepancy is to assume that many of the smaller proteins seen on the gel pattern are either intermediate or final breakdown products of large molecular weight precursors, and

indications that such is the case have come from Summers and Maizel (1968) and Jacobson and Baltimore (1968 a). Both groups have been able to detect a loss in radioactive label from the largest protein NCVP1 and a concomittant rise in peaks corresponding to smaller virion proteins during the course of a pulse-chase experiment. Such metabolic instability for NCVP1 can be prevented by the addition of amino acid analogues to infected cells, suggesting that cleavage of NCVP1 occurs by an enzyme (or enzymes) with specificity for certain amino acid sequences (Jacobson and Baltimore, 1968 a).

The phenomenon of cleavage has been noted by Holland and Kiehn (1968) for a number of picornaviruses and by Burrell, Martin and Cooper (1969) in cells infected with Semliki Forest virus, a group A arbovirus.

Jacobson and Baltimore (1968 a) have suggested that post-translational cleavage is an adaptive feature of the growth of simple RNA viruses in animal cells, which allow no possibility of internal protein chain termination and initiation. In more complex RNA viruses such as influenza (Duesberg, 1968 a) and reovirus (Bellamy and Joklik, 1967), the genome consists of several short pieces of RNA, an arrangement which may obviate the necessity for cleavage. In paramyxoviruses such as Newcastle disease virus, which

contains one very large RNA molecule, the actual genetic message consists of several short complementary RNA strands (Bratt and Robinson, 1967). Whether ancillary cleavage mechanisms exist in cells infected with these latter viruses is unknown.

A.3.6 Assembly and maturation

Viral RNA, although first detectable within 30-60 minutes, does not enter mature virions until 2.5 hours after infection (Baltimore et al., 1966). These authors also found that the time for synthesis of RNA and entry into virions is relatively short, of the order of 2-3 minutes, although an average time of 20 minutes must elapse between the synthesis of protein and its incorporation into completed particles. This implies a larger pool of viral protein than viral nucleic acid precursors. Another finding of Baltimore et al. (1966) was that assembly of capsid polypeptides into virions can occur in the absence of continuing protein synthesis. This is demonstrated by the observation that viral RNA which is synthesised several minutes after the addition of cycloheximide, an inhibitor of protein synthesis, can still enter virions.

Jacobson and Baltimore (1968 b) have suggested that one of the final steps in the maturation of poliovirions is

the cleavage of a large precursor polypeptide, termed VPO or NCVP6 (Summers et al., 1965), into two smaller components of the completed virion (VP2 and VP4). Cleavage appears to occur prior to the entry of a molecule of viral RNA to produce the mature virion and may be blocked with guanidine, leading to the formation of particles devoid of RNA, but containing the precursor polypeptide VPO and defective in their content of VP2 and VP4. Such particles are similar to the 'top component' noted previously in populations of poliovirus particles by Hummeler, Anderson and Brown (1962) and Scharff and Levintow (1963). A similar profile for the 'top' component polypeptides was described by Maizel, Phillips and Summers (1967).

B. Genetic Studies with Poliovirus

B.1 The aims and methods of genetic analysis

A genetic analysis seeks to delineate different aspects of the viral growth process from atypical features of the replication of variant or mutant strains of a parental virus type. An obvious requirement in studies of this kind is the need for considerable understanding of the normal growth process and, in this respect, poliovirus is a worthy object of study, being probably the best characterised of all animal viruses. Thus one approach in a genetic analysis is to obtain a series of mutants of the parental

virus and to group them, physiologically, according to common differences each member of the group exhibits in its replication.

Such an approach is aided considerably by tests of complementation and recombination between different pairs of mutants, which assist the experimenter to decide whether a certain physiological property of a mutant virus is attributable to specific characters or markers in one or more genes.

In a complementation test, cells are infected with pairs of viral mutants. If markers exist in separate genes, then each mutant may reciprocally provide the functional gene products specified by the non-marker genes of its own genome, in order to produce an enhanced yield of unmutated parental virus. Complementation, therefore, involves no interchange of original genetic material, but occurs as an apportionment of gene products at the post-translational level. In systems where efficient complementation is known to occur, failure to complement may be accepted, in conjunction with other tests, as evidence that both markers are present in a single gene. Such an interpretation, however, is rendered difficult when complementation occurs within a single gene.

The best indication of gene order is provided by recombination studies, whereby a cell is infected with two mutant strains of a virus and an interchange of genetic material takes place. Among the final yield of infectious progeny virus then are copies of a hybrid genome, consisting of unmutated parts of the genetic material of either parental mutant. The further apart are the genetic markers, the greater is the probability that interchange of genetic material will occur. The actual frequency of recombination between different markers allows for a very good indication of their order, and hence of the genes they represent, on the viral genome.

In the bacteriophage T4D an extension of the above mentioned methods of genetic analysis involves an attempt to relate, <u>in vitro</u>, the interaction of gene products specified by different segments of the virus genome in the morphogenesis of the completed particle (Edgar and Wood, 1966). When studies of this kind are realised the ultimate goal of genetic analysis will have been largely achieved: namely, the development of a map of the genome specifying the number of genes present, their order on the virus genome, and the detailed functional relationships which they bear to one another and to the apparatus of the host cell. A study of this kind would provide the fullest possible account of replication for that particular virus.

B.2 The genetic coding potential of poliovirus

The RNA genome of poliovirus is small in relation to other animal viruses and consists of a continuous length of ribonucleic acid whose molecular weight is slightly in excess of 2×10^6 daltons (see experimental section 3). This is equivalent to about 6,000 nucleotides and in comparison with the well characterised RNA bacteriophage MS2, whose RNA has a molecular weight of 1.1×10^6 (Strauss and Sinsheimer, 1963) and specifies three gene products (Gussin, 1966; Tooze and Weber, 1967), the coding capacity for poliovirus should be of the order of six genes. Such comparisons, however, may not be applicable to poliovirus, because of the apparent lack of specific mechanisms for internal protein chain initiation and termination in poliovirus RNA (see previous section).

Summers et al. (1965) noted the synthesis of four virion proteins and a maximum of ten non-virion proteins in cells infected with poliovirus. The function of these non-virion proteins is completely unknown, but included among them is probably at least one polymerase and a protein which is responsible for the inhibition of cellular macromolecular synthesis. It is possible that some virion proteins may fulfil these functions and that most non-capsid proteins are non-functional precursors

of virion proteins. Some evidence for a polymerase function which is associated with the virion proteins of foot-and-mouth disease virus, another picornavirus, was presented by Polatnick and Arlinghaus (1967). They were able to specifically inhibit the activity of viral polymerases from infected cells with antisera prepared against purified virions.

B.3 Earlier genetic studies with poliovirus

The importance of an understanding of the genetics of poliovirus was realised in the 1950's with the development of attenuated (non-neurovirulent) strains for use in mass vaccination. Understandably, the genetic character most widely selected against then was that of neurovirulence for primates, but, because of technical difficulties in testing for this property on a large scale, a variety of other characters or markers, empirically related to neurovirulence, were developed. Some of these markers were subsequently applied in standard genetic tests and examples of them include the ability to plaque under agar at acid pH (the *d* marker; Vogt, Dulbecco and Wenner, 1957), capacity to multiply at 40°C (the *rct* 40 marker; Lwoff and Lwoff, 1959 a and b), sensitivity to dextran sulphate inhibitor (Takemoto and Leibhaber, 1962)

and thermolability at 50°C (McBride, 1962). A comprehensive list of poliovirus markers has been presented by Cooper (1969).

Very few of these markers are representative of parts of the genome other than those specifying the property of virion proteins to adsorb to cell receptors. Others, such as the property of thermolability, appear to result from mutations at any of several different loci in one gene (McCahon, 1967). An overlying complication with many markers, which may explain the failure of earlier workers to achieve recombination, is the observation (Dulbecco, 1961; Cooper et al., 1966) that mutants from different parts of the genome exhibit co-variation, whereby a particular phenotype is shared by mutants with defects in several genes.

Markers, not associated with neurovirulence and which are apparently not co-variant, were used by Hirst, (1962) and Ledinko (1963) in the first demonstration of recombination between strains of poliovirus. These are markers specifying resistance to horse and bovine serum inhibitors and to guanidine (markers ho, bo and g). By doubly infecting HeLa cells with strains ho and bo, Hirst obtained double mutants containing both markers at about fifteen times the frequency which was observed in

self-crosses. The actual frequency of recombination was about 0.37 per cent.

Ledinko (1963) extended these findings using strains containing the ho marker, which had been adapted to thermostability (the Δ^r marker). In crosses between strain hoΔ^r and another strain specifically adapted to guanidine resistance (strain g), she obtained heat-stable recombinants with the g and ho character. Such three-factor crosses allowed a marker order of g-ho-Δ^r to be assigned to the viral genome and Hirst (private communication to P.D. Cooper, 1967) in other crosses has established the sequence g-ho-bo for both serum inhibitors and guanidine. Markers g and bo were roughly equidistant from ho.

Later studies (Cooper, 1968) suggest that these markers represent those parts of the poliovirus genome coding for virion proteins, which have a combined molecular weight of 92,500 (Maizel and Summers, 1968) out of a total amino acid coding potential of 236,000 (this assumes that the genome specifies 2,000 amino acids, each with an average molecular weight of 118.0 (Levintow and Darnell, 1960)).

Far more useful for genetic tests of poliovirus are the temperature sensitive (ts) mutants developed by Cooper

(1964 c), which have the potential to be induced in any gene by individual base substitutions and are directly related to functional defects in different stages of the viral growth process. Their use and the potential of other kinds of conditional lethal mutants will be dealt with in the following sections.

B.4 Conditional lethal mutants for use in 'total' mapping studies of viruses

For a 'total' genetic analysis viral mutants are required with markers representative of all parts of the genome and conditional lethal mutants appear to fulfil this requirement adequately. With these mutants the actual genetic marker is a defect in the viral genome caused by specific alterations to component nucleotides, which may result in a mutant being unable to replicate in a particular host cell under certain conditions of growth. There are two classes of conditional lethal mutants, the suppressor sensitive (su) mutants and the temperature sensitive (ts) mutants.

Conditional lethal mutants of both kinds have been used to great effect in mapping and identifying over half of the genes of the DNA bacteriophages T4 (Epstein, Bollé, Steinberg, Kellenberger, Boy de la Tour, Chevalley, Edgar, Susman, Denhardt and Leilausis, 1963) and λ (Campbell 1961).

Both kinds of mutants have also been noted for RNA bacteriophages (Zinder, 1965).

Suppressor sensitive mutants have been found only in bacteriophages and their existence is due to the codon recognising apparatus of bacterial cells in bringing about the termination of protein synthesis at intervals along the RNA messenger. Thus in normal (su) cells, changes in codons for amino acid recognition to other (non-sense) codons, such as UAG, UAA or UGA, may be sufficient to bring about the premature termination of polypeptide synthesis and so to give rise to non-functional proteins (Brenner, Stretton and Kaplan, 1965). Such alterations may be caused by single base changes and termination arises from the lack of a species of charged transfer RNA with the appropriate anticodon recognition site for the altered triplet.

In other (su^+) hosts, transfer RNA with anticodon specificity exists but, instead of chain termination, another amino acid is inserted, which leads to the formation of a slightly altered, though still functional, gene product. In su^+ cells this alteration in codon recognition is due to changes in the region of the bacterial genome which codes for transfer RNA (Stretton and Brenner, 1965), although here it is necessary to postulate a separate chain terminating mechanism to specify the normal completion of each gene product.

The other category of conditional lethal mutants are the temperature sensitive (ts) mutants. These arise from base substitutions that produce a gene product, which unlike the parental strain, is either unable to assume or maintain a particular functional configuration at elevated temperatures of viral growth. In some instances, the occurrence of a mutation in a particular gene can be correlated with the in vitro lability of the corresponding gene product. Examples of this are mutants of the bacteriophage T4D in the gene for viral DNA polymerase (de Waard, Paul and Lehman, 1965) and of bacteriophage λ in the gene for lysozyme (Campbell and del Campillo-Campbell, 1963).

With bacteriophages, mutants of both kinds may arise spontaneously from mistakes in the assembly of viral nucleotides, or as a result of the action of mutagens. With animal viruses ts mutants only have been obtained for poliovirus (Cooper, 1964 c), Sindbis virus (Burge and Pfefferkorn, 1964), polyoma virus (Fried, 1965) rabbitpox virus (Sambrook, Padgett and Tomkins, 1966), Semliki Forest virus (Tan, Bellett and Sambrook, 1969), influenza virus (MacKenzie, 1968), vesicular stomatitis virus (Pittman, 1965), Newcastle disease virus (Kirvaitis and Simon, 1965) and Reovirus (Fields and Joklik, 1969). The lack of

evidence for true suppressor sensitive mutants with animal viruses may be related to the presumed absence of codons for internal protein chain termination in animal messengers.

B.5 Studies with ts Mutants of poliovirus

Cooper (1964 c) was able to isolate a number of ts mutants of poliovirus by growing a parental strain in the presence of the base analogue 5 - fluorouracil (5-FU) which is incorporated into the RNA genome in place of uracil (Munyon and Salzman, 1962). The mutagenic action of 5-FU is due to its ability to occasionally assume an enol configuration, due to the loss of a proton at position 1 of the pyrimidine ring. Where base pairing occurs, as it does at several stages of the replication of poliovirus RNA (section A-3.3e), the enol state of 5-FU has the specificity of cytosine and changes in the complementary purine will take place in the synthesis of positive or negative strands (Hayes, 1964). About thirty mutants were obtained with 5-FU which were able to multiply at 37.2° C (the optimum temperature for the growth of the parental strain ts^+) but not at 39.2-39.6° C.

B.5.1 Genetic interactions between *ts* mutants of poliovirus

Cooper (1965) examined various pairs of these mutants for complementation and was able to achieve an enhancement of yield between *ts*-5 and *ts*-19 some fourteen times the sum of individual mutant yields. This cooperative or marker rescue effect was shown to be asymmetric, with only *ts*-19 being detectable in the final yield. However, complementation was relatively low (a maximum of 0.1 per cent of the wild type yield) for this and other pairs of mutants tested and McCahon (1967) suggests that inefficient complementation may be due to interference between mutants with the growth of strain ts^+ at the restrictive temperature. Interference with the growth of ts^+ by *ts*-19 at restrictive temperatures was first demonstrated by Pohjanpelto and Cooper (1965).

Genetic recombination between various pairs of *ts* mutants was shown by Cooper (1968). By analysis of the factors causing variability, he developed a standardised test which allowed reproducible map distances to be assigned to crosses between various mutant pairs. A number of genetic sequences were obtained by three-factor crosses between certain mutants and the mutant *ts*-28 which had been adapted to resistance to guanidine (*ts*-28g). Mutants having single genetic defects were shown in reciprocal

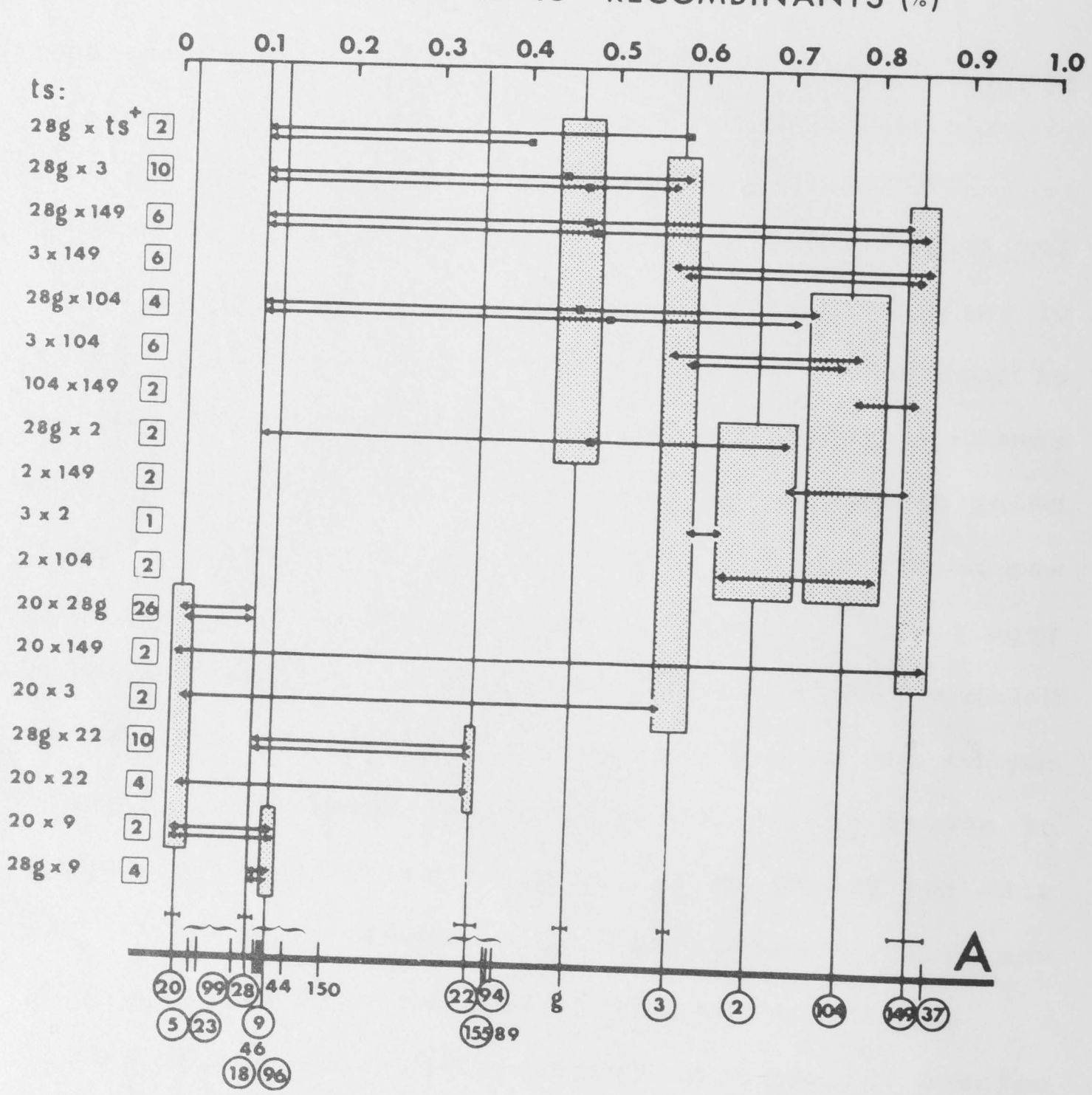


Figure 1: Genetic map of poliovirus ts mutants (Cooper, 1968). Mean ts+ recombination frequencies from those crosses showing additivity. The values in the squares are the number of crosses made. The upper of two frequencies for a particular cross represents the standardized, and the lower the unstandardized, frequency; a single frequency is the standardized one, except that no standards were available in the tests made for the cross 20 x 22. The map at A (ts-28 being arbitrarily fixed at 0.1 per cent) represents the relative positions of those mutants showing additivity, together with others (ts-46, -96, -44, -150, -94, -155, -89) whose positions have only been determined in crosses with ts-28 g, and with the loci of the major defects of some double mutants (ts-5, -23, -99, -18, -37). The lengths of the horizontal bars at the positions of some main mutants represent the 95 per cent confidence limits for the means of their self crosses. The relative position of loci within a bracketed group is uncertain.

crosses to give additive recombination frequencies and to provide the basis of the genetic map shown in Figure 1. A maximum recombination frequency of 0.85 per cent was obtained.

Many original mutants were excluded from the map because of the presence of more than one genetic leison in their genome. Reversion frequencies at the restrictive temperature were of little use in deciding whether a particular mutant was single or not, although the revertant plaque character did provide some indication. The best indication of singularity was the demonstration of additivity in recombination frequencies in various reciprocal crosses, and on this basis only mutants ts-2,-3, -28 and -104 could be unequivocally accepted as having a single defect. Mutants ts-20 and ts-149 occurred at extremities of the map and hence could not be tested for additivity, but in other respects were like single mutants. Still other mutants could not be used because of relatively high growth ('leak') at restrictive temperatures.

The locus for guanidine resistance with mutant 28g could be mapped accurately and appeared to lie in the region of the genome which coded for virion proteins. This finding was unexpected in view of earlier findings that guanidine

inhibited the synthesis of viral RNA and viral RNA polymerase activity (Eggers, Reich and Tamm, 1963; Baltimore, Eggers, Franklin and Tamm, 1963).

Subsequent work has confirmed the association between resistance to guanidine and changes in virion protein structure (Cooper, Wentworth and McCahon, 1969). They showed that six out of eight mutants with apparently single defects in the region of the g locus were even more sensitive to guanidine than the parental strain ts^+. The possibility that the virion protein or proteins which determine resistance to guanidine merely suppress a primary site of sensitivity in another gene (the phenomenon of genetic dominance) was deemed unlikely by the demonstration that the growth of a guanidine-resistant strain was inhibited by a sensitive strain in the presence of an inhibitory concentration of guanidine.

Support for the association between virion proteins and guanidine resistance also comes from the capacity of certain mutants (ts-19, -22, -89, -123 and -149), with defects adjacent to the g locus, to interfere with the growth of ts^+ at 39.5^{o} C (McCahon, 1967). A reduced yield of infectious RNA is produced as a result of interference, which suggests that defects in the conformation of virion proteins are determinants of viral RNA synthesis.

Table 1

Summary of results of physiological tests of poliovirus *ts* mutants [a]

Physiol. group	ts	Physiological test and test temperature			
		a^+ 39.4°	ppi_3^+ 39.6°	pti^+ 39.6°	Critical period of cycle
parental	+	+	+	+	none
A	5	-	-	-	late
	20	-	-	-	late
	23	-	-	-	early
	81	-	-	-	late
	99	-	-	-	ND
B	18	-	-	+	ND
	22	-	-	+	late
	28	-	-	+	late
	44	-	-	+	ND
	46	-	-	+	late
	104	-	-	+	late
	123	-	-	+	ND
C	19	-	+	+	late
	37	-	+	+	late
	89	-	+	+	late
D	2	+	+	+	ND
	3	+	+	+	ND
	8	+	+	+	ND

a. extract from Table 1 of Cooper, Johnson and Garwes (1966) from McCahon (1967).

b. a^+ = production of antigen; pti^+ = prevention of incorporation of thymidine; ppi_1^+, ppi_2^+, ppi_3^+ = prevention of phosphorus incorporation into RNA fractions 1, 2 and 3, respectively.
In the body of the table, + = wild-type response:
- = markedly defective:
ND = not determined.

B.5.2 Physiological studies with *ts* mutants

About twenty of the original *ts* isolates were grouped physiologically into four groups which corresponded with different gene functions (Cooper, et al., 1966). Three mutants *ts*-2, -3 and -8 produced antigen detectable by a fluorescent antibody test (the *a* marker). Five mutants were found to be defective in their capacity to inhibit cellular DNA, as judged by the ability of the infected cell to incorporate radioactive thymidine (the *pti* marker), and this property was shown to be related to a prevention of cellular uptake of trypan blue. Other mutants were defective in their ability to inhibit the 4-10S species of cellular RNA (the *ppi3* marker). The *a*, *pti* and *ppi3* markers appeared to covary asymmetrically in a manner which is illustrated in Table 1.

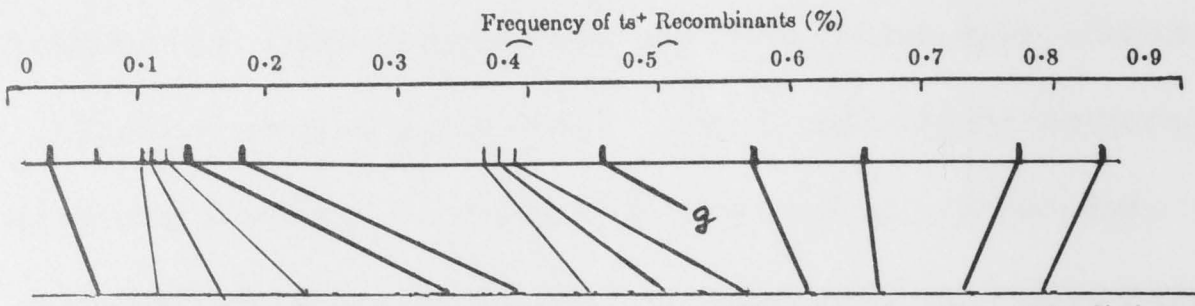
Temperature shift experiments revealed that all mutants were defective in functions which appeared 'late' in the virus growth cycle except *ts*-23, which has since been shown to possess a multiple defect.

Subsequently, all mutants were examined for the production of infectious RNA and for serum-blocking antigen. Yields of infectious RNA varied from less than 0.5 per cent to 88 per cent of the yield of the parental strain (Wentworth, McCahon and Cooper, 1968). Large

producers of RNA were associated with the right hand side of the genetic map and, with some exceptions, low producers were associated with the left. The exceptions (ts-19, -22, -89, -104 and -149) also have defects in virion proteins and, as mentioned previously, such mutants are capable of interfering with the growth of ts^+ at 39.6°C (McCahon, 1967). Studies, in which the amount of serum-blocking antigen produced by these mutants under restrictive conditions was measured, gave essentially similar results.

The interpretation of such experiments is often rendered difficult by an unusually high production of viral mutants at the restrictive temperature ('leak' virus). Under such conditions, the absolute yield of infectious RNA or viral antigen is not necessarily a reliable indication of the nature of the genetic defect. Thus the infectious RNA produced by a number of mutants under restrictive conditions was assessed in terms of its maturation efficiency, whereby the amount of infectious RNA was related to the amount of virus produced, in comparison to the situation with parental ts^+ (Wentworth et al., 1968).

On the basis of maturation efficiency, a clear distinction could be made between mutants with defects occurring on the right and the left of the map, with the



ts^+	20	81	28	46	96	44	150	94	89	155	3	2	104	149	Single mutants
	A	A	B	B	B	B	·	C	C	C	D	D	B	·	Group
+	+	+	+	+	+	+	·	+	+	+	+	+	+	·	ppi_1
+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	*pti*
+	−	−	−	−	−	−	·	+	+	+	+	+	−	·	ppi_3
+	−	−	−	−	−	−	−	−	−	−	+	+	−	·	*a*
100	·09	3·4	2·9	3·0	·	7·4	22	60	6·8	44	34	88	3·3	3·1	i RNA
+	+	·	+	+	+	+	+	+	−	+	+	+	+	−	Interference
+	·	+	+	+	·	+	+	−	−	−	−	−	+	−	Maturation efficiency
+	+	+	+	+	+	+	+	−	+	−	−	−	−	+	Δ 45°C
+	+	+	+	+	+	+	+	−	−	−	−	−	+	+	*cy*
+	+	+	+	+	+	+	+	−	−	−	+	−	+	−	*g*/120

RNA Synthesis

Virion Protein

Table 2: Summary of recombination data and tests of physiological function for mutants of poliovirus with single ts defects (Cooper, 1969). g represents the locus of guanidine inhibition; ppi1 and ppi3 = prevention of phosphorus incorporation into cellular RNA fractions 1 and 3; pti = prevention of thymidine incorporation; a = antigen synthesis, i RNA = infectious RNA production in relation to ts^+; interference = capacity to interfere with the growth of ts^+g at 39.5°; maturation efficiency = proportion of infectious RNA converted to mature virions in relation to ts^+; Δ45° C = thermostability at 45° C; cy = cystine requirement for virus growth; g/120 = inhibition by guanidine at 120μg/ml.

sole exception of ts-104. Other tests such as those concerned with the in vitro stability of mutants at 45° C and their capacity to produce plaques in media containing low concentrations of L-cystine suggest that mutants to the right (i.e. those in the vicinity of the g locus) have defects in the assembly of virion proteins (McCahon, 1967; Cooper, 1969). A summary of these findings for various single mutants in relation to the earlier findings of Cooper et al. (1966), and to the location of a particular defect for each mutant on the genetic map is shown in Table 2.

C. The Determination of the Molecular Weight of Viral RNA

Where attempts are made to correlate known molecular events in the growth of a virus in terms of total genetic function, an accurate estimate of the molecular weight of the viral nucleic acid is essential. For the RNA of poliovirus a value of 2.0×10^6 daltons is widely accepted (Schaffer, 1962; Fenner, 1968), but Cooper (1969) has drawn attention to inadequacies of data in support of this figure. Because of this, further attempts were made to assess the molecular weight of poliovirus RNA and this work is reported in Experimental Section 3. In the following section a brief outline is given of some of the methods

available for determining the molecular weight of viral RNA preparations.

C.1 Estimates based upon sedimentation coefficients

A number of empirical formulas have been proposed that relate RNA sedimentation coefficients to molecular weight (Gierer, 1958; Kurland, 1960, Spirin, 1961) and by far the greater number of determinations have been obtained in this manner. Such relationships, however, take no account of different amounts of secondary structure that exist between various RNA species. An example of this can be seen from RNA preparations of tobacco mosaic virus (TMV) and the RNA bacteriophage R17; both have identical sedimentation coefficients, yet the RNA of TMV had a molecular weight almost twice that of R17, when determined by the accurate light scattering technique (Gesteland and Boedtker, 1964). R17 RNA is a much more compact macromolecule than TMV RNA because of a relatively high degree of internal base pairing (Boedtker, 1967). A high degree of secondary structure is also present in the RNA of Sindbis virus (Sprecher-Goldberger, 1967). In the case of the RNA from certain tumour viruses, a further complication arises from a tendency of the RNA to sediment homogeneously as an aggregate structure Duesberg (1968 b).

The removal of RNA secondary structure

When RNA secondary structure is eliminated after heating in the presence of formaldehyde (Boedtker, 1968 a) or treatment with 99 per cent dimethyl sulphoxide (DMSO) at room temperature (Strauss, Kelly and Sinsheimer, 1968) a linear relationship has been observed between molecular weight and sedimentation coefficient. Fenwick (1968) in a study using formaldehyde-treated RNA preparations, noted a similar type of relationship for all RNA species except 28S HeLa ribosomal RNA. The latter with a molecular weight of 1.65×10^6, when determined by equilibrium centrifugation and viscosity studies, was shown to have a sedimentation coefficient greater than that of TMV (2.0×10^6) after formaldehyde treatment. Fenwick has suggested that this anomalous behaviour of 28S ribosomal RNA may be due to inherent differences in its structure compared with that of TMV RNA; cross-linkages may arise in 28S RNA as a result of formaldehyde treatment, or the RNA may occur naturally as a branched structure. Such behaviour, however, was not noted by Strauss et al. (1968) for rabbit reticulocyte RNA after treatment with DMSO.

C.2 Molecular weight determinations by light scattering

For RNA molecules with molecular weights of 100,000 or greater, light scattering provides a very accurate and

rapid method of determining molecular weights over a large range of temperature and salt concentrations. An outstanding advantage of the method is that it allows for a determination of molecular weight without the need for assumption as to the shape of the molecule or its size distribution. A review of theoretical and technical considerations involved has been given by Boedtker (1968 b).

The phenomenon of light scattering is basically one of diffraction and, since scattered light has for the most part the same wave-length as that of incident light, it can be easily distinguished from ordinary fluorescence. Useful information is obtained by measuring relative intensity, depolarisation and the angular distribution of scattered light. These quantitites are then analysed in terms of the electromagnetic theory of radiation and the kinetic theory of matter, according to modifications of the basic equation of Rayleigh (1871) derived by Doty and Edsal (1951) for dilute protein solutions.

There are two major disadvantages of the method. First, very large quantities of RNA are needed. Boedtker (1968 b) estimates that about 3 mg are needed for measurements of an RNA of molecular weight 1.0×10^6, over a fivefold concentration range. Concentrations of this order are attainable only with extreme difficulty with most

animal viruses and, as a consequence, the only data available have been obtained from the RNA of plant viruses and RNA bacteriophages. The second major difficulty arises from the presence of very small concentrations of highly aggregated material which may be present in concentrated RNA solutions. The presence of aggregates is readily recognisable when the scattering data are analysed according to the method of Zimm (1948). Aggregates may be removed by repeated centrifugation of dilute RNA solutions.

C.3 Sedimentation-viscosity measurements

This technique, although principally used for determining the molecular weights of DNA samples, has been applied in estimating the RNA components of mammalian ribosomes (Petermann and Pavlovec, 1966), and of poliovirus (Anderer and Restle, 1964). Molecular weights are determined from a knowledge of the sedimentation coefficient of an RNA preparation and its intrinsic viscosity in dilute salt solutions, applying the formula of Scharaga and Mandelkern (1953).

However, as for the sedimentation coefficient mentioned in C.1, the intrinsic viscosity is partly dependant upon the particular conformation assumed by an RNA preparation, which varies with different RNA species to a different extent at different ionic strengths (Boedtker,

1968 b). There is thus no single equation relating molecular weight with sedimentation-viscosity measurements for all types of RNA. A value of 2.0×10^6 for type II poliovirus RNA was obtained by Anderer and Restle (1964) but the particular sedimentation coefficient of 28.2S that they used was less than the now generally accepted value of 35 S for type I poliovirus RNA (Baltimore and Girard, 1966).

C.4 Equilibrium ultracentrifugation

Results obtained by this technique, as with light scattering, are not influenced by RNA secondary structure and large amounts of preparative material are not required. A possible disadvantage is that with large RNA molecules, centrifugation for up to several days is often required before equilibrium can be attained. Although this time may be shortened considerably by the use of smaller centrifugation cells, a consequent loss of precision often results (Boedtker, 1968 b). Equilibrium ultracentrifugation has been used to provide estimates of the molecular weights of E. coli and mammalian ribosomal RNA components (Stanley and Bock, 1965; Hamilton, 1967).

C.5 Polyacrylamide gel electrophoresis

High resolution in the fractionation of RNA species was attained by Loening (1967) by electrophoresis on dilute polyacrylamide gels. Bishop, Claybrook and

Spiegelman (1967) achieved similar resolution in another study and were able to demonstrate a linear semi-logarithmic relationship between molecular weight and relative electrophoretic mobility. Separation of RNA by this method is likely to be related to the pore size of the gel, as differences in the ratios of charge to mass of different RNA molecules are considered likely to be too small to affect their relative electrophoretic mobilities (Loening, 1967). Because of the influence of pore size, secondary structure is likely also to be a determinant of electrophoretic mobility, and evidence for this is presented in Experimental Section 3. The method is thus open to the same objections mentioned earlier for estimates of molecular weight based upon sedimentation constants, in which no attempt was made to remove RNA secondary structure.

MATERIALS AND METHODS

1. Cell Culture Preparation

(a) Cell propagation

Human amnion strain U cells (Pohjanpelto, 1961) were used throughout except in one experiment (see Experimental Section 2.1 (h)) where the HeLa cell line S3, adapted for growth in monolayer cultures, was used. Conditions of culture were identical for both cell lines.

About 6×10^6 cells were added to Roux bottles, containing 100 ml of Eagle's medium with 10 per cent tryptose phosphate broth, 10 per cent calf serum and 0.7 mg per ml sodium bicarbonate, pH 7.2. Confluent monolayer growth occurred during 4-6 days of incubation at 37° C. The cells were then detached from the glass by treatment with 20 ml of a solution, consisting of 0.25 per cent trypsin and 0.01 per cent sodium ethylene diamine tetracetic acid (EDTA), in Hank's balanced salt solution (Hanks and Wallace, 1949), for 5 minutes at room temperature. Galactose conservator medium (GCM; Cooper, 1961) was then added and the cells removed by centrifuging at low speeds. They were finally suspended at 6.0×10^6 cells per ml in GCM. Generally a 4-6 fold increase in viable cell numbers occurred over the period of cell cultivation.

(b) Tube monolayer cultures of U cells

These were used in recombination tests and for the propagation of progeny from suspected mutant plaques. Stoppered flat bottomed glass vials of internal diameter 17 mm were seeded with 2.0×10^5 U cells in 1 ml of Eagle's medium, containing 10 per cent tryptose phosphate broth, 5 per cent calf serum and 0.28 mg per ml sodium bicarbonate, pH 7.2, and were incubated overnight at 37° C in an upright position.

(c) Bottle cultures

These were used in the preparation of virus stocks. A suspension of 2.0×10^6 freshly trysinised U cells in Eagle's medium containing 10 per cent tryptose phosphate broth, 10 per cent calf serum and 0.28 mg per ml of sodium bicarbonate, pH 7.2 was added in 10 ml amounts to milk dilution bottles. Confluent cell growth resulted when the bottles were incubated in a horizontal position overnight at 37° C.

(d) Suspension cultures and their use in virus studies

Freshly trypsinised cells, suspended in GCM at 6.0×10^6 cells per ml, were rocked at 4° C for 2-3 hours in order to regenerate cell receptors. The suspension was centrifuged at low speeds and the cells resuspended at about 1.0×10^7

cells per ml in phosphate buffered saline (PBS; Dulbecco and Vogt, 1954). Virus at the appropriate concentration was then added and was allowed to adsorb while the suspension was rocked at 4^{o} C for 2-3 hours.

After infection, the cells were diluted to a concentration of 1-2 x 10^{6} cells per ml in Eagle's medium, containing 5 per cent calf serum, 5 per cent tryptose phosphate broth and 0.7 mg per ml sodium bicarbonate, pH 7.2. The cultures were placed in stoppered spinner culture flasks with rotating magnets, and were placed in constant temperature waterbaths mounted on magnetic stirrers. The suspension was kept agitated by use of the stirrers for the time of the experiment. Any cell receptors that were destroyed by trypsinisation were apparently restored by rocking in GCM at 4^{o} C, as these cells were shown to be as susceptible to poliovirus as other cells grown in suspension at 37^{o} C for 18 hours before use.

(e) Eagle's medium

This was prepared according to the formula of Eagle (1959), except that double the concentration of amino acids and vitamins were used. Penicillin and streptomycin were added at a concentration of 50 µg per ml, and sodium bicarbonate, calf serum and tryptose phosphate broth were

added as required at concentrations indicated elsewhere in this section. For suspension cultures, Eagle's Spinner Medium was used which differed from normal Eagle's medium in that it contained no calcium chloride.

(f) Temperature control

A 37° C laboratory incubator was used for the routine propagation of cells and for virus and RNA infectivity assays at permissive temperatures. For spinner cultures and assays of infectivity at 39.5° C, well circulated waterbaths were used and these were heated by thermostatically controlled units (Manufacturers:- E. Buhler, Tubingen; B. Braun, Melsungen) that were accurate to ±0.05° C.

2. Infectivity Assays

(a) Assays of poliovirus

The agar-cell suspension assay (Cooper, 1961) was employed throughout, using suspensions of U cells in GCM at initial concentrations of 6.0×10^6 cells per ml. To the galactose overlay medium used for assays was added 8 per cent calf serum, 5 per cent of a 10 per cent suspension of skim milk powder and 10 per cent tryptose phosphate broth. In assays of ts^+ recombinants at 39.5° C, lactalbumin hydrolysate and yeast extract were omitted from the medium, in order to reduce the 'leak' rates of

cystine dependant ts mutants (Cooper, 1968). Analar guanidine carbonate solution, adjusted to pH 7-8, was added at either inhibitory (200 µg/ml) or non-inhibitory (10 µg/ml) concentrations in the media used for the assay of ts^+g and ts^+ recombinants, respectively. The low guanidine concentration was used to satisfy a slight guanidine dependance of the mutant ts-28g that was used in most crosses.

For normal assays, dilutions of virus were made in PBS and 0.1 ml amounts were assayed by the standard technique. For assays of viral recombinants, virus was diluted initially in 0.05 M glycine buffer, pH 2.5 and then neutralised in order to eliminate aggregation as a source of variation and to increase sensitivity (Fenwick and Cooper, 1962). Virus assays at 37^o C were conducted in 2 inch Petri dishes, and, at 39.5^o C, in screwcapped aspirin bottles which were immersed in a well circulated 39.5^o C waterbath.

(b) Calculation of recombination frequency

The recombination frequency is taken as the proportion of ts^+ or ts^+g in the total yield of virus, i.e. the ratio of titres obtained from assays at 39.5^o C compared with titres obtained at 37^o C (Cooper, 1968). This proportion is adjusted, where necessary, to allow for

reversion to ts$^+$ or ts$^+$g by either parental strain, and for any guanidine dependence in strain ts-28g.

(c) Screening of plaque progeny for ts mutants

In the screening of the progeny from relatively large numbers of suspected mutant plaques, rapid tenfold dilutions to 10^{-4} were made in PBS with calibrated loops by a serological microtechnique (Sever, 1962). Two-drop samples of each dilution were then assayed for virus at 37^{o} and 39.5^{o} C, using a single plate and a single bottle, respectively, and both were stained in the usual manner (Cooper, 1959) after 3 days. By this method the progeny of up to 100 plaque isolates could be tested simultaneously.

(d) RNA infectivity titration

A modification of the cell suspension infectivity assay of Koch, Quintrell and Bishop (1966) was used in assays of poliovirus infectious RNA. Ten millilitres of a suspension of U cells (6×10^6 cells per ml in GCM) were washed twice with PBS and resuspended in PBS minus calcium and magnesium salts. Just prior to use, 5 ml of this suspension was mixed with 5 ml of PBS (minus calcium and magnesium) containing 100 μg per ml DEAE-dextran. This was the standard infectious RNA diluent, ID. The resultant suspension of sensitised cells was then diluted 20 fold

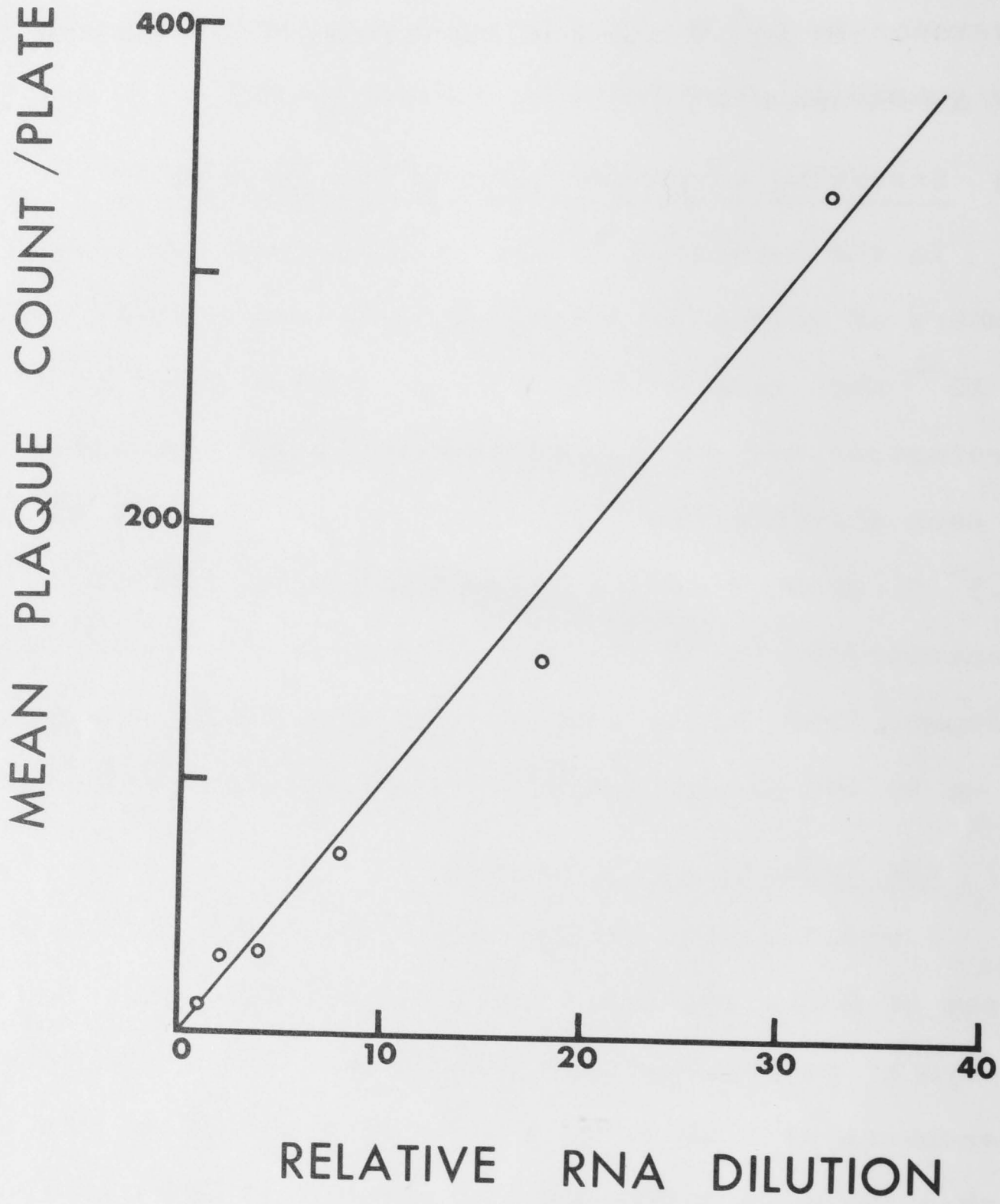


Figure 2: The relationship between plaque numbers and relative RNA concentration. A closely spaced series of dilutions of ts$^+$ infectious RNA was made in ID and the infectivity of replicate samples was determined by the agar-cell suspension technique described in Materials and Methods.

with PBS, minus calcium and magnesium salts, and kept at 4^{o} C until used.

For assays, dilutions of infectious RNA were made in ID and 0.1 ml aliquots were mixed with 0.6 ml of sensitised cells, and incubated at 37^{o} C for 5 minutes. One tenth of one millilitre of ID was then added and the mixture was incubated at 37^{o} C for an additional hour, with occasional shaking. Finally, 0.6 ml of a freshly prepared suspension of U cells (6×10^{6} cells per ml in GCM) and 1.0 ml of galactose agar medium (Cooper, 1961) were added and, after rapid agitation, the mixture was poured onto an agar base layer in a 3 inch Petri plate. Plates were stained after three days of incubation at 37^{o} C and any plaques that were present were counted. The method was found to give more reproducible results than a similar assay in which U cell monolayer cultures were infected directly with dilutions of infectious RNA.

In an attempt to establish that each RNA plaque was initiated by a single infectious unit, a closely spaced series of dilutions of strain $\underline{ts}^{+}$ RNA was made and duplicate assays of each dilution were carried out as described above. The results of the experiment are presented in Figure 2, which demonstrates a linear relationship between plaque numbers and relative RNA

concentration. This suggests that each plaque was initiated by a single unit of infectious RNA and such a relationship is a criterion for the validity of quantitative infectivity assays (Dulbecco, 1952). Titres for infectious RNA by the cell suspension method were about 10^{-2}-10^{-3} of those obtained in assays of unextracted whole virus.

(e) Poliovirus strains used

These were derived from a Sabin vaccine strain (VS41) that had been adapted by Dr A. Lwoff to grow at 41° C. Strain ts^{+} was obtained by Dr P.D. Cooper from a plaque in a 40° C assay, and is referred to as the parental or 'wild type' strain.

For much of the work to be described mutants previously isolated by Cooper (Cooper, 1964 c) were used, and these were mutants of the series ts-2 to -155 that were obtained by the growth of ts^{+} in the presence of the mutagen 5-fluorouracil (5-FU). Two series of isolations were made. In the first, a mutant isolation rate of 10 per cent was attained after the growth of ts^{+} in the presence of 1 m M 5-FU, and the mutants included ts-2 to -23. Mutants ts-28 to -155 were isolated at a rate of 2-3 per cent in the presence of 0.25 m M 5-FU, the lower dose of mutagen being used in order to minimise the chance of obtaining

double mutants. After recloning twice, about 30 mutant stocks had efficiencies of plating at 39.5° C that were sufficiently low for the strains to be used in genetic studies (Cooper, et al., 1966).

(f) Preparation of stocks from newly isolated mutants for use in recombination tests

Details of the procedure used for the isolation and screening of plaque progeny for the presence of ts mutants are given in Experimental Section I or in Section 2(c) of Materials and Methods. In the preparation of stocks of suitable mutants for use in recombination tests the procedure of Cooper (1968) was followed.

Small stocks of low ts^+ revertant content were prepared by infection of tube cultures containing 10^5 cells with the maximum amount of a cloned mutant stock which would allow less than 1 tube in 20 to receive 1 plaque forming unit (p.f.u.) of ts^+. After growth for 2-3 days at 37° C, these stocks were assayed for ts^+ revertants at 39.5° C and for the efficiency of plating at 39.5° C compared with 37° C (e.o.p. $_{39.5^o}$). Stocks with e.o.p. $_{39.5^o}$ values less than 10^{-4} and with a smaller revertant content were used to infect three monolayers of 5×10^6 U cells in milk dilution bottles. After growth for 8 hours in a 37.2° C waterbath, the cultures were frozen

and assayed for *ts*$^+$ content at 39.5^o C. Stocks with *ts*$^+$ contents of 10^{-4} or less were then selected, and prepared for use in recombination tests as follows. The stocks were clarified by centrifuging for 20 minutes at 200 r.p.m. and the virus was then sedimented by centrifuging for 90 minutes at 39,000 r.p.m. in a Spinco 40 rotor. After standing overnight at 4^o C in 0.5 ml of 0.9 per cent sodium chloride solution, the pellets were resuspended, treated with 1 ml of 0.05 M glycine buffer and neutralised after 3 minutes with 10 ml Eagle's medium. The virus was then recentrifuged at 39,000 r.p.m. and the pellet resuspended by standing overnight in 1 ml Eagle's medium containing 0.5 per cent calf serum. The concentrates were pooled, re-clarified, dispensed in 0.2 ml amounts in ampoules and stored at -60^o C. Titres were 5-10 x 10^8 p.f.u. per ml.

Virus stocks when prepared by this method ('recombination stocks') behaved much more uniformly in recombination tests than untreated preparations (Cooper, 1968).

(g) Virus stocks for use in polymerase experiments

Four day old Roux cultures (containing about 2.4 x 10^7 cells) were infected with 0.5 ml of a preparation of mutant recombination stock and, after growth at 37^o C for 1-2 days, the culture was frozen. After thawing, samples of 2-3 ml (virus titre 3-5 x 10^8 p.f.u. per ml) were placed

in vials and stored at -15° C.

(h) Standard recombination test

Again the procedure of Cooper (1968) was closely followed. Cells from semi-confluent 2-4 day Roux bottle cultures were suspended to 2.5×10^5 cells per ml in Eagle's medium containing 10 per cent tryptose phosphate broth, 7.5 per cent calf serum, 2.5 per cent foetal calf serum, 1 mg per ml calcium chloride and 0.77 mg per ml sodium bicarbonate, pH 7.2. The medium had been previously gassed with 5 per cent carbon dioxide in air and 1 ml of the cell suspension was added to stoppered flat bottomed tubes and incubated for 20 hours at 37.2° C.

The resultant monolayer was washed twice with PBS and the fluid present thoroughly aspirated. The cells were then infected with 0.08 ml of virus inoculum prepared from fresh ampoules of recombination stock, diluted to 1.5×10^8 p.f.u. per ml in Eagle's medium. In test crosses equal volumes of this suspension were mixed before inoculation, giving an input multiplicity of about 24 p.f.u. per cell. For self-crosses, undiluted suspensions of each mutant were used to infect tube cultures. Each cross was conducted in duplicate and each duplicate was assayed separately, using two plates or bottles per dilution, with

appropriate self-crosses and control cultures infected with $\underline{ts}^+$ and $\underline{ts}^+$g.

The inoculated tubes were restoppered and shaken at 10-15° C for 4 hours to allow for maximum adsorption, and then 0.92 ml of Eagle's medium containing 100 µg/ml of cycloheximide, 10 per cent tryptose phosphate broth and 5 per cent calf serum pH 7.2, was added and the cultures placed in a 37.2° C waterbath for 20 minutes. The cultures were then chilled and washed twice to remove unadsorbed virus, and 1 ml of Eagle's medium, containing 10 per cent tryptose phosphate broth, 5 per cent calf serum and 0.28 mg per ml sodium bicarbonate, pH 7.2 was added and the tubes incubated for an additional 9 hours at 37.2° C. They were then frozen at -15° C until assays of the virus present could be carried out.

3. The Preparation of RNA

RNA preparations were obtained by modifications of the basic phenol extraction technique, which are described as follows:

(a) Preparation of infectious RNA

A suspension of 1-2 x 10^8 U cells was infected with strain $\underline{ts}^+$ at multiplicities in excess of 3 p.f.u. per cell, and was grown in spinner culture for 8-10 hours at 37.2° C.

Cells from the suspension were removed by centrifugation and were resuspended in about 10 ml of PBS, minus calcium and magnesium salts.

RNA was prepared from infected cells by adding an equal volume of twice distilled phenol, containing 0.01 M EDTA and bentonite (1 mg per ml) and shaking at 60° C for 5 minutes. Bentonite was prepared according to the method of Fraenkel-Conrat, Singer and Tsugita (1961). The mixture was centrifuged at 3,000 r.p.m. for 10 minutes and the aqueous upper RNA layer removed with a Pasteur pipette and extracted twice more. One tenth volume of 2M sodium chloride and two volumes of ethanol (AR grade) were then added and an RNA precipitate that formed over two hours at 0° C was collected by centrifugation. The precipitate was dissolved in a small volume of PBS (minus calcium and magnesium salts) containing 0.01 M EDTA, and, after an additional precipitation, was dissolved again in PBS and stored in sealed glass ampoules at -45° C.

(b) Extraction of RNA from polymerase reaction mixtures for sucrose gradient analysis

Polymerase reaction mixtures were shaken at 60° C for 5 minutes with an equal volume of phenol, containing 1 per cent sodium dodecyl sulphate (SDS), 0.01 M EDTA and bentonite (1 mg per ml). The

mixture was then chilled and centrifuged at 3,000 r.p.m. for 10 minutes and the aqueous RNA was removed and kept at 0^{o} C until required for use.

(c) Preparation of RNA for use in studies on the molecular weight of viral RNA

Because of the need to obtain non-degraded preparations of RNA for use as viral markers, the more gentle single-phase phenol extraction method of Diener and Schneider (1968) was used in the extraction of RNA from purified virions. This involved the use of a reagent which was prepared by adding 0.4 ml of phenol to 1.5 ml of a solution containing 3 per cent SDS and 0.01 M EDTA in 0.02 M phosphate buffer, pH 7.0. At this concentration phenol was soluble in the reagent.

Equal volumes of a purified virion suspension and the phenol reagent were mixed and allowed to stand at room temperature for 15 minutes. Sixty micrograms of an aqueous preparation of purified yeast RNA was then added to each mixture and the total RNA present was precipitated as described above. The RNA precipitate was collected by centrifugation and dissolved in a small volume of the appropriate buffer, before subjecting the sample to electrophoresis on polyacrylamide gels or to sucrose gradient centrifugation.

4. Sucrose Gradients and Their Use in Analytical Studies of RNA

Linear sucrose gradients were prepared in four steps by layering solutions containing decreasing concentrations of sucrose in NET buffer (0.1 M sodium chloride, 0.01 M EDTA and 0.01 M Tris (hydroxymethylaminomethane) buffer, pH 7.4). The complete operation was carried out in a 4° C cold room and the centrifuge tubes were covered and kept in an upright stationary position for 18 hours at 4° C, to allow equilibration within the various sucrose layers. The gradients were used as required and were then fractionated.

For gradients prepared for use in a Spinco SW-39 centrifuge head (prepared by four 1.1 ml layering steps), a fractionating device described by Schaffer and Frommhagen (1965) was used, in which the tubes were punctured at the bottom and 0.2 ml amounts displaced through the bottom of the tube by the use of a screw micrometer. These gradients were either fractionated directly onto 4 x 3 cm strips of Whatman no.1 filterpaper according to the method of Dalgarno, Martin, Liu and Work (1966), or were collected in tubes for further treatment before adding to the papers. SW-25 gradients (prepared by four 6.6 ml layering steps) were fractionated after centrifugation by puncturing and collecting 16 drop fractions into tubes. Aliquots of these fractions were then placed on paper strips as required.

The papers were dried and treated with gentle agitation for 5 minutes with 7 changes of ice cold 0.25 M perchloric acid containing 0.9 per cent sodium pyrophosphate. They were then subjected to two 10 minute changes of cold ethanol before being rinsed twice with ethanol/ether (1:1) and ether. After drying, the papers were transferred to scintillation vials containing 16 ml of fluid consisting of 0.5 per cent 2,5-diphenyloxazole (PPO) and 0.05 per cent 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (POPOP) in toluene (AR grade).

The acid insoluble radioactivity present on each paper was determined in a Packard series 3,000 scintillation counter. For the simultaneous counting of ^{14}C and ^{3}H two channels were used, with window settings of 210-850 and 50-1000 and corresponding gains of 11 and 50 per cent. These settings were checked each time by reference to standards containing a single isotope.

5. The Use of Polyacrylamide Gel Electrophoresis in Analytical Studies of RNA

Modifications of the techniques of Loening (1967) and Bishop, Claybrook and Spiegelman (1967) were used in the preparation of 2.4 per cent polyacrylamide gels in 'Perspex' tubes (internal diameter 7 mm). The gels were supported at one end of the tube by a dialysis membrane. Acrylamide was

purified for use in gels by dissolving in chloroform at 50° C, filtering and crystallising at -15° C. The crystals were washed with cold chloroform, dried and stored under vacuum. Bisacrylamide was purified by dissolving in acetone at 40-50° C, filtering and crystallising in the same manner.

Four ml of a stock solution consisting of 15 per cent (W/V) acrylamide and 0.75 per cent bisacrylamide (W/V) were mixed with 12.45 ml of distilled water and 8.33 ml of electrophoresis buffer 3E (0.06 M sodium acetate, 0.003 M EDTA and 0.012 M Tris, pH 7.2). Air was removed from the mixture by evacuation and 50 µl of 10 per cent SDS was added followed by 50 µl of N,N,Ń,Ń-tetramethylethylenediamine (Temed) and 50 µl of 10 per cent (W/V) ammonium persulphate. The contents were mixed rapidly and were added to the 5 or 7 cm level of 7 or 9 cm tubes.

The tubes containing the gels were held upright and polymerisation occurred after 15-20 minutes at room temperature. They were then transferred to a standard electrophoresis apparatus, such as was described by Davis (1964), with 500 ml of buffer E (3E diluted 1:2) containing 1 per cent SDS in either reservoir. Electrodes were attached and direct current was applied at 70 volts and 6-7 mA per tube for 30 minutes in order to remove

ribonuclease from the gels. Sucrose to 10 per cent and one drop of bromphenol blue marker was then added to RNA samples, whose preparation has been described, and 70-80 µl of each sample was layered on top of a gel and electrophoresed.

After electrophoresis the gels were removed from the tubes, and frozen in a metal trough on a block of dry ice. They were then allowed to thaw slightly before fractionating simultaneously with a device consisting of a parallel series of razor blades set at 1.4 mm apart between the teeth of two combs. Individual slices were transferred in sequence to empty scintillation vials, to which was added 0.5 ml of 10 per cent (V/V) piperidine. The slices were dried by placing the vials in a 60° C oven overnight and 0.5 ml of distilled water was then added, and the slices allowed to swell to 10-20 times their original volume with occasional rocking.

Ten millilitres of scintillation fluid consisting of 18 per cent naphthalene (scintillation grade), 0.8 per cent PPO and 0.01 per cent POPOP in dioxan (AR grade) was then added to each vial. The addition of the fluid caused the slices to shrink and freed about 90 per cent of radioactive material from the gel (Loening, 1967). Radioactivity was determined with similar window settings and gains on the scintillation counter to those used in the gradient experiments.

6. Procedures Used in the Determination of Viral Polymerase Activity

(a) Preparation of polymerase from infected cells

Suspensions of 1-2 x 10^8 infected U cells in 100 ml of medium were incubated in spinner culture at temperatures and for times given in Experimental Section 2. The cells were then chilled and removed from the suspension by low-speed centrifugation. They were resuspended in 50 ml of Polymerase Diluent (0.25 M sucrose, 10^{-3} M magnesium chloride) and stored at -15° C until required for enzyme extraction.

The extraction procedures were adapted from those of Baltimore and Franklin (1963) and Tershak (1966). Cells were removed from Polymerase Diluent, after thawing, by centrifugation and were resuspended in 20-40 ml of ice cold distilled water and the suspension was kept in an ice bath for 1 hour. The cells were then disrupted by treating the suspension with 5-10 strokes of a 40 ml Potter-Elvehjem tissue homogeniser and the suspension examined microscopically to ensure that cell disruption was complete. Nuclei and cell membranes were removed by centrifuging at 600 g for 10 minutes and magnesium chloride and Tris-hydrochloric acid were added to the supernatant cytoplasmic extract to give final concentrations of 5 x 10^{-3} and 0.1 M respectively, and a pH of 7.6. One tenth volume of 5 M sodium chloride was then added and the mixture left for

5-10 minutes at 0^{o} C. During this time a viscous precipitate of nucleoprotein appeared, and the viscosity was relieved by diluting twofold with cold distilled water. The total extract was then centrifuged at 20,000 r.p.m. (35,000 g) for 2 hours in a Spinco model L ultracentrifuge using a type 30 angle rotor.

After centrifugation the supernatant was discarded and the pellet washed twice with Polymerase Diluent. It was finally broken up and suspended in 1-2 ml of Polymerase Diluent with the aid of a ground glass tissue homogeniser. The final suspension had a protein concentration of 1.5-2.5 mg per ml, as determined by the method of Lowry, Rosebrough, Farr and Randall (1951), using standards prepared from bovine plasma albumin (Armour fraction V).

Addition of sodium chloride is a necessary step in the procedure and where omitted the activity of the preparation was reduced by about two thirds (unpublished observation). Its significance is unknown.

(b) Standard polymerase assays

Reaction mixtures of 0.8 ml were prepared in 15 ml conical centrifuge tubes and contained 0.2 ml of enzyme, 50 mμmoles of ATP, UTP and CTP, [^{3}H] GTP (20 mμmoles; 100 c/M), 6 μmoles 2-phosphoenol pyruvate, 6 enzyme units (E.U.)

pyruvate kinase, 5 µmoles magnesium acetate, 1.3 µg AMD and 30 µmoles Tris-hydrochloric acid, pH 8.4. Where the RNA from reaction mixtures was examined on sucrose gradients or in polyacrylamide gels, 0.2 ml of enzyme was incubated in a reaction mixture consisting of the same reagents plus 1 mg bentonite in a final volume of 0.53 ml.

In standard polymerase assays the reaction was stopped after incubation by the addition of 0.5 ml of 0.1 M sodium pyrophosphate, followed by 5 ml of 10 per cent (W/V) trichloracetic acid (TCA). The mixture was left for 10 minutes at 0^{o} C for a precipitate to form and was then centrifuged at 3,000 r.p.m. for 10 minutes. The supernatant was discarded and 0.5 ml of 0.5 N sodium hydroxide, 0.5 ml of 0.1 M sodium pyrophosphate and 5 ml of cold TCA were added in rapid succession. The precipitate was centrifuged again and washed twice with cold 10 per cent TCA and once with ethanol/ether (1:1). It was dissolved in 1 ml of formic acid and the solution transferred to scintillation vials containing 20 ml of a fluid consisting of (per litre) 770 ml toluene, 230 ml ethanol, 4 g PPO and 0.25 g POPOP. Radioactivity was determined in the scintillation counter using appropriate standards for [^{3}H] GTP.

7. Radioactively Labelled Viral and RNA Marker Preparations

(a) Poliovirus particles

A suspension of 1-2 x 10^8 U cells was infected at a multiplicity of 3-5 p.f.u. per cell, and was then incubated as a 100 ml spinner culture at 37.2° C. After 3 hours, actinomycin D was added to a final concentration of 1 µg per ml followed, after 15 minutes, by 20 µc of uridine-2-^{14}C (specific activity 20-50 c/M). Incubation at 37.2° C was continued until 8-10 hours when the culture was chilled and the cells removed by light centrifugation. They were suspended in about 20 ml of PBS and disrupted by two cycles of alternate freezing and thawing. Nuclei and cell membranes were removed by centrifuging at 1000 r.p.m. for 15 minutes and the virus present in the supernatant was sedimented by centrifuging at 39,000 r.p.m. for 90 minutes using a Spinco 40 angle rotor.

The pellets were rinsed and resuspended in 0.5 ml of PBS after standing for 2-3 days at 4° C. Aliquots of the suspension of 0.2 ml were then purified by centrifuging through pre-formed 40-48 per cent caesium chloride gradients for 6 hours at 35,000 r.p.m. using a Spinco SW-39 rotor. Fractions of 0.1 ml of the gradient were collected into tubes, and those containing the peak of infectivity and radioactivity were pooled and subjected

to a second gradient centrifugation. The peaks were again pooled and caesium chloride removed from the suspension by dialysing overnight against PBS. The suspension was stored at 4^{o} C until required for use.

(b) Preparation of RNA from poliovirus infected cells

A spinner culture consisting of poliovirus infected U cells was set up at 37.2^{o} C as in 7(a), and AMD and uridine-2-^{14}C were added in the same manner at 3.0 and 3.25 hours, respectively. Incubation at 37.2^{o} C was continued until 6 hours, when the suspension was chilled and the cells removed by light centrifugation and resuspended in 5-10 ml of PBS (minus calcium and magnesium salts). RNA from these cells was then extracted with phenol/SDS at 60^{o} C, as described in 3(a), and was precipitated twice with ethanol before being dissolved in 1-2 ml of NET buffer. The suspension was stored at -45^{o} C until required for use.

(c) Preparation of Replicative Form RNA

One ml of the poliovirus RNA preparation obtained in 7(b) was treated with 10 μg of ribonuclease for 30 minutes at 25^{o} C. The mixture was then centrifuged through preformed 15-30 per cent sucrose gradients (26.4 ml) at 18,000 r.p.m. for 16 hours using a Spinco SW-25.1 rotor. Fractions were collected and those containing the peak

of acid insoluble radioactivity, which was resistant to ribonuclease, were pooled and stored at -45° C.

(d) U cell ribosomal marker RNA

An uninfected suspension culture, consisting of 3×10^8 U cells in 100 ml of spinner medium, was incubated at 37.2° C for 18 hours in the presence of 30 μc of uridine-2-^{14}C (specific activity 50 c/M). The cells were removed from suspension by light centrifugation, and were resuspended in 5-10 ml of NET buffer. RNA was extracted by incubating three times with phenol/SDS at 37° C for 5 minutes. After centrifugation, RNA in the aqueous phase was precipitated twice with ethanol and was finally redissolved in 10 ml of NET. Aliquots of 0.5 ml were then transferred to vials which were stored at -45° C.

(e) Tobacco mosaic virus (TMV)

A sample of [^{3}H] TMV of high activity was obtained from Dr A.J. Gibbs, and had been prepared in the following manner:-

The virus was grown in leaves of the tobacco plant Nicotiana tabacum L. after manually inoculating the leaves with a mixture of TMV and diatomaceous earth, the latter being used as an abrasive. The type strain U1 of TMV was used, and infected plants were kept in a glass house at

20-24° C. After 5 days when symptoms of infection first became apparent, the leaves were detached from the plant and slices were made under water with a razor blade to produce 5 mm ribbons at right angles to the main midrib of the leaf, leaving the margins of the leaf and the main midrib still intact.

Excess moisture was removed and the leaves placed in Petri dishes, where they were treated with several drops of a solution containing 100 μc/ml of [^{3}H] adenosine (specific activity 4,000 c/M). After several hours, a minimal volume of water was added to keep them moist and the Petri dishes were exposed to indirect sunlight over an additional 2 days at 25° C.

The virus was extracted by grinding the leaves in borate buffer (0.005 M sodium borate, 0.018 boric acid, pH 7.0). The suspension was then subjected to low speed centrifugation to remove cellular material, and the virus present was sedimented by centrifuging at 39,000 r.p.m. for 2 hours in a model L ultracentrifuge using a 40 rotor. The virus pellet was suspended in a small volume of borate buffer and finally purified by centrifuging for 2.5 hours at 23,000 r.p.m. through pre-formed 10-40 per cent sucrose gradients, using an SW-25 rotor. Fractions were collected and those in the region of the peak of radioactivity were pooled and stored at 4° C.

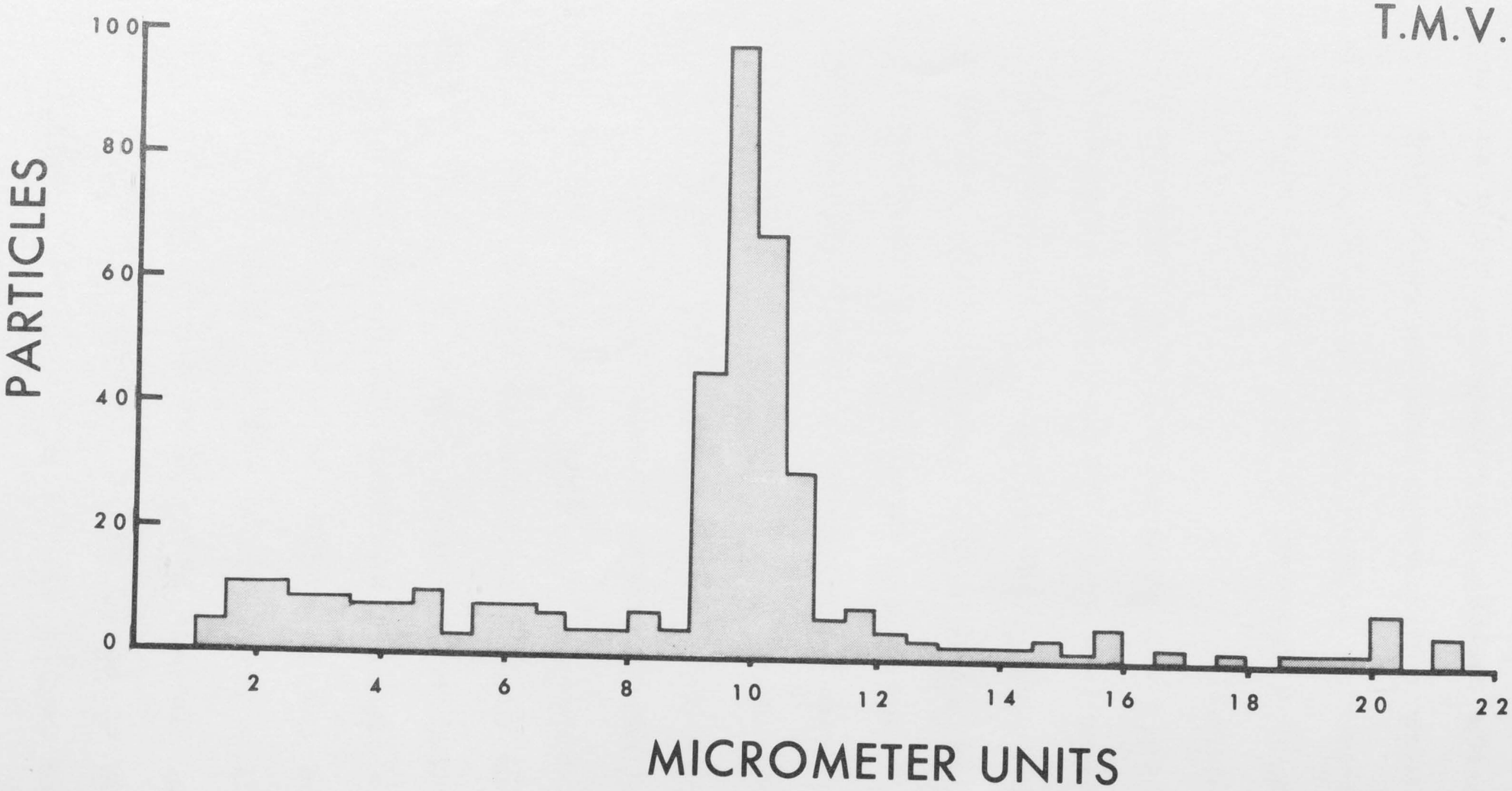


Figure 3: Distribution of particle lengths in a suspension of [^{3}H] TMV. Images from electron micrographs of a suspension of [^{3}H] TMV were obtained after negative staining with sodium phosphotungstate. Particle lengths were determined with a micrometer eye piece and are expressed as arbitrary units.

The virus suspension was examined for particle length homogeneity by mixing an aliquot of the purified suspension with neutral 4 per cent sodium phosphotungstate and spraying onto the carbon film of an electron microscope grid. After drying, the preparation was examined in a Siemens-Elmiskop electron microscope and 'photographs' were made of the preparation at a fixed instrument magnification of 20,000. Images from several micrographs were used to measure particle lengths, using a micrometer eye piece, and the type of distribution that was obtained is shown in Figure 3. Clearly the suspension contained particles of uniform length, and this is supported from data on the homogeneity of TMV RNA after extraction in Experimental Section 3. If extensive shearing of particles had occurred during preparation, the extracted RNA would be heterogeneous in its sedimentation behaviour and thus unsuitable for use as a marker in molecular weight determinations.

(f) RNA Bacteriophage R17

A purified suspension of [^{3}H] R17 was obtained from Dr A.J. Gibbs. It was prepared from a virus stock supplied by Dr A.J.E. Colvill and was grown and assayed in *E. coli* hfr met$^-$ male bacteria, according to procedures outlined by Watanabe and August (1967). The initial virus seed

was prepared from infected cells that were grown in LB medium (Loeb and Zinder, 1961) whereas, in the preparation of radioactively labelled bacteriophage medium 121 (Hershey, 1955), was used.

Two hundred microcuries of [^{3}H] adenosine (4,000 c/M) was added to an infected 2 litre culture, containing 2×10^8 cells per ml, throughout the time of viral growth. The cells were lysed and the virus present was purified according to the method of Watanabe and August (1967). The final preparations contained 10^{11}-10^{12} p.f.u. of bacteriophage.

(g) Semliki Forest virus (SFV)

Purified suspensions of [^{3}H] SFV were obtained from Dr C.J. Burrell and were prepared as follows:

A suspension of 3.5×10^8 BHK cells were infected with the Kumba strain of SFV at a multiplicity of 5-10 p.f.u. per cell. After adsorption at 4° C, the cells were grown in spinner culture at 37° C in medium that consisted of modified Eagle's medium (MacPherson and Stoker, 1962), containing 10 per cent tryptose phosphate broth, 10 per cent calf serum and 0.28 mg per ml sodium bicarbonate. The medium had been gassed with 5 per cent carbon dioxide in air to give a final pH of 7.2

After 3.5 hours, 100 μc of [^{3}H] adenosine (4,000 c/M) was added and incubation at 37° C was continued until 9 hours. Cellular material was then removed by low-speed centrifugation and the non-viral protein present in the supernatant was partly removed by adding 0.8 ml of protamine sulphate to give a final concentration of 1 mg per ml. After shaking, the mixture was allowed to stand at 4° C in the dark for 2.5 hours and the precipitate was removed and discarded after centrifuging at 3000 r.p.m. for 15 minutes.

Virus present in the supernatant was then further purified by two centrifugations through pre-formed 30-50 per cent (W/W) sucrose gradients at 23,000 r.p.m. for 16 hours using an SW-25.1 rotor. The gradients were prepared in buffer consisting of 0.1 per cent calf serum, 0.1 M potassium chloride and 0.01 M Tris-hydrochloric acid, pH 9.0. After each centrifugation fractions containing the peak of radioactivity and infectivity were collected and were stored at 4° C until required for use. The identity of the radioactive preparation was then checked, after negative staining, by examining under the electron microscope. Titres of about 10^9 p.f.u. per ml were obtained in this manner, when assayed in chick embryo fibroblasts.

8. Sources of Chemicals

Chemical	Source
[^{3}H] Guanosine-5′-triphosphate [^{3}H] Adenosine-5′-triphosphate [^{3}H] Uridine-5′-triphosphate Uridine-2-^{14}C	Schwarz Bio Research, Inc. Orangeburg N.Y., U.S.A.
[^{3}H] Adenosine	The Radiochemical Centre, Amersham, U.K.
Adenosine-, Guanosine-, Cytidine- and Uridine-5′-triphosphate (ATP, GTP, CTP and UTP)	Sigma Chemical Co., St Louis Mo., U.S.A.
Cycloheximide	
Pyruvate kinase 2-phosphoenol pyruvate	Calbiochem., Los Angeles, Calif., U.S.A.
Pancreatic ribonuclease	
Acrylamide Bisacrylamide	Eastman Organic Chemicals, Rochester, N.Y., U.S.A.
'Temed'	
Formaldehyde (36% W/V; AR)	British Drug Houses Ltd., Poole, U.K.
Dimethyl Sulphoxide	
Lithium chloride	

Chemical	Source
Piperidine Napthalene Toluene (AR)	British Drug Houses Ltd., Poole, U.K.
PPO POPOP	Packard Instrument Co. Inc., La Grange, Illinois, U.S.A.
Dioxan (AR)	Ajax Chemicals Ltd., Sydney, Australia.
Snake venom phosphodiesterase	Worthington biochemical Corp., Freehold, N.J., U.S.A.
Puromycin dihydrochloride	Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.
Caesium chloride (optical grade)	Trona, American Potash and Chemical Corp., Los Angeles, U.S.A.
Guanidine carbonate	Fluka, A.G. Switzerland

EXPERIMENTAL SECTION 1

THE ISOLATION OF ADDITIONAL ts MUTANTS OF POLIOVIRUS

Mutants obtained so far with 5-FU may be broadly grouped into those having defects in viral maturation which occur to the right of the genetic map and those with defects in the replication of viral RNA which occur on the left. Just how representative of the complete genome is the map shown in Figure 1 is unclear, but among the functions which do not appear to be represented are those concerned with the inhibition of cellular 28S and 18S RNA and protein synthesis in response to infection. Thus attempts were made to obtain additional ts mutants of poliovirus which are more representative of the extremities of the map, using mutagens other than 5-FU. In one series of experiments described below an exonuclease, snake venom phosphodiesterase, was used in an attempt to induce terminal ts deletion mutants at the 3′-terminus of the genome. In other experiments the mutagenic properties of nitrous acid were studied.

1.1 Studies with Snake Venom Phosphodiesterase

This enzyme acts by removing individual nucleotides sequentially from the 3′-terminus of DNA or RNA polynucleotides (Razzell, 1963), although in earlier

preparations traces of endonuclease activity were detectable (Razzell and Khorana, 1959). Earlier studies by Holland, McClaren, Hoyer and Syverton (1960), had indicated that poliovirus infectious RNA could be inactivated with snake venom phosphodiesterase and a linear type of inactivation was seen, indicative of single-hit kinetics. In the same paper, poliovirus RNA was shown to be insusceptible to the action of E. coli alkaline phosphatase, which suggests that the terminal 3′-phosphate is not required for infectivity. The concept behind the work to be described was to treat poliovirus RNA with a sufficiently low concentration of venom phosphodiesterase in order to remove some terminal nucleotides; it was hoped that such a procedure would leave the remainder of the RNA molecule still infectious, but would produce a ts defect in the particular protein specified by the gene adjacent to the 3′-terminus.

If poliovirus replication is similar to that of the RNA bacteriophages, a deletion of this kind would induce a defect in the viral polymerase. Two lines of evidence support this. First, the direction of synthesis of viral RNA and protein is from the 5′ to the 3′ RNA terminus (August, Banerjee, Eoyang, Franze de Ferdinandez, Hasegawa, Hori, Kuo, Rensing and Shapiro, 1968) and, secondly, the

order of translation of the gene products is, first, viral coat protein, then maturation factor and, finally, viral RNA polymerase (Spahr and Gesteland, 1968; Lodish, 1968).

Analagous information is lacking for poliovirus and nothing is known of the specificity of the viral polymerase for the 3′ terminal nucleotides in the replication of viral RNA. It is possible that terminally deleted molecules of viral RNA may be non-functional. Kamen (1969) has observed that the 3′ terminal adenylate of bacteriophage R17 may be removed without destroying viral infectivity, whereas the removal of the proximal cytidylate does destroy infectivity. The following experiments were conducted before many of the details concerning RNA bacteriophage replication became available.

Incubation mixtures were set up at 37^{o} C, consisting of 0.1 ml of infectious poliovirus RNA, 0.1 ml of enzyme, and 0.8 ml of substrate diluent (0.01 M ammonium acetate, 0.3 M magnesium acetate, 0.1 M Tris-acetate buffer, pH 8.8). A control mixture was prepared consisting of the same constituents but with PBS (minus calcium and magnesium salts) in place of enzyme. Concentrations of enzyme of 3×10^{-5} and 3×10^{-6} units per ml were used in each incubation mixture and the reaction was stopped by diluting one hundred fold in RNA diluent ID (see

Materials and Methods). Surviving infectious RNA was assayed by a replicate dilution series, using the cell-suspension assay described in Materials and Methods.

The infectivity assay was adapted as a selective procedure for use in isolating ts mutants. Assays were carried out using 4 inch Petri plates (large enough to accommodate up to 200 discrete plaques) and these were incubated for one day at 37° C to allow the initiation of plaque formation by all the infectious RNA present in the assay sample. The plates were then transferred to 39.5° C for an additional two days before spraying with INT stain to reveal plaques. By this procedure it was hoped that plaques from unmutated parental virus would be much larger than the mutant plaques with ts defects. Counts were made of all plaques which were present on each plate and suspect mutant plaques were picked with a Pasteur pipette and transferred to separate one day old U cell tube cultures. Three days were allowed for plaque virus multiplication to occur before the cultures were frozen and stored at -10° C. The progeny from each isolate could be later tested for ts characteristics, according to the procedure described in Materials and Methods.

Three experiments were conducted in this manner and the kinetics of inactivation obtained from one experiment

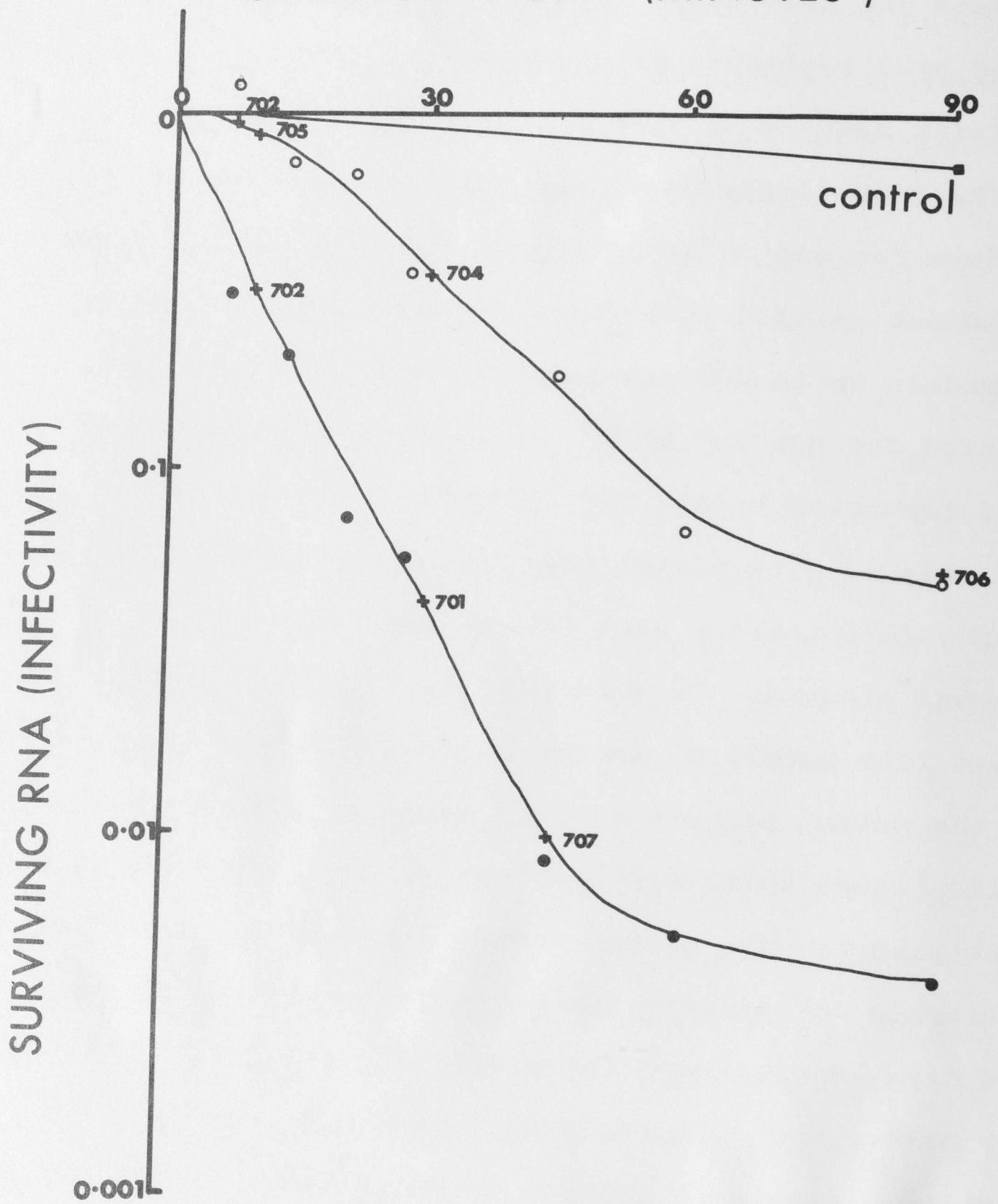


Figure 4: The kinetics of inactivation of ts^{+} poliovirus infectious RNA by snake venom phosphodiesterase. Mixtures of infectious RNA, enzyme and substrate diluent were prepared as described in the text and were incubated at 37° C for varying times. The reaction was stopped by diluting in ID and surviving infectious RNA was determined by the agar-cell suspension assay. Final enzyme levels of either 3×10^{-6} units (closed circles) or 3×10^{-7} units (open circles) were used in two series of reaction mixtures. Mutants obtained under different conditions of incubation are indicated (+).

are shown in Figure 4. Superimposed on Figure 4 are the various ts mutants obtained from all three experiments under various conditions of enzyme degradation.

In Figure 4 a rapid, apparently single-hit type of inactivation occurs during the early stages of inactivation with the more concentrated enzyme preparation. Using the more dilute preparation, a slight shoulder can be seen in the earlier parts of the curve, which is suggestive of a 'multi-step' type of inactivation. Such an inactivation may allow for some degradation of proximal nucleotides without affecting viral infectivity. A flattening appeared in both curves after approximately 60 minutes of incubation, which may be due to aggregation or to secondary structures in a fraction of the RNA molecules, rendering them unsusceptible to further degradative attack. This flattening was not noted by Holland et al. (1960), although their maximum time of incubation was only 30 minutes.

As the total number of mutants isolated is relatively small and these are isolated from plates containing varying plaque numbers, it is difficult to define the actual mutant isolation rate for a particular set of

experimental conditions. However, it is possible to assign a total isolation rate for each experiment and this varied from 10 per cent, in the experiment in which ts 701, 702 and 703 were isolated, to 1-2 per cent for the remaining two experiments. The occurrence of mutants in Figure 4 fails to indicate any particular set of conditions of incubation more favourable for their isolation, although the number of isolates is small. Both isolation rates appear to be considerably in excess of the spontaneous mutation rate for poliovirus of around 0.1 per cent, a figure derived largely from mutant reversion frequencies (P.D. Cooper, personal communication). A total of seven ts mutants was obtained and the best of these were prepared for use in recombination tests. The results of recombination tests will be discussed later in conjunction with a single nitrous acid mutant.

1.2 Studies with nitrous acid

Nitrous acid is a known mutagen for poliovirus (Boeyé, 1959; Carp and Koprowski, 1962), which acts upon the RNA genome in vitro by deaminating three of its component bases: adenine, guanine and cytosine are converted to hypoxanthine, xanthine and uracil respectively (Schuster and Schramm, 1958). In base pairing situations, hypoxanthine is specific for cytosine as is xanthine,

despite the absence of a third hydrogen bond in forming the base pair (Hayes, 1964). Thus, nitrous acid has a broader capacity to bring about base changes than 5-FU, although, as a consequence, many interactions will probably have multiple and lethal effects upon the capacity of the altered RNA molecule to undergo replication. Whole virus is inactivated at about three times the rate as infectious RNA, due to changes in virion proteins which are brought about by deamination (Boeyé, 1959, 1962) and, therefore, for mutant isolations it was considered best to treat infectious poliovirus RNA with nitrous acid.

Two types of isolation procedure were used. In the first, infectious RNA was treated with an equal volume of nitrous acid (prepared from a 1:1 mixture of M sodium acetate and 4M sodium nitrite, pH 4.5) at room temperature (20^{o} C). At various intervals the reaction was stopped by diluting one hundred fold in RNA diluent. Assays of surviving infectious RNA were then conducted on four inch Petri plates, and the temperature shift procedure during incubation described above for venom phosphodiesterase experiments was used in attempts to differentiate mutant from unmutated parental plaques. Plaques were counted and small plaques were picked and their progeny grown up as in the previous experiment.

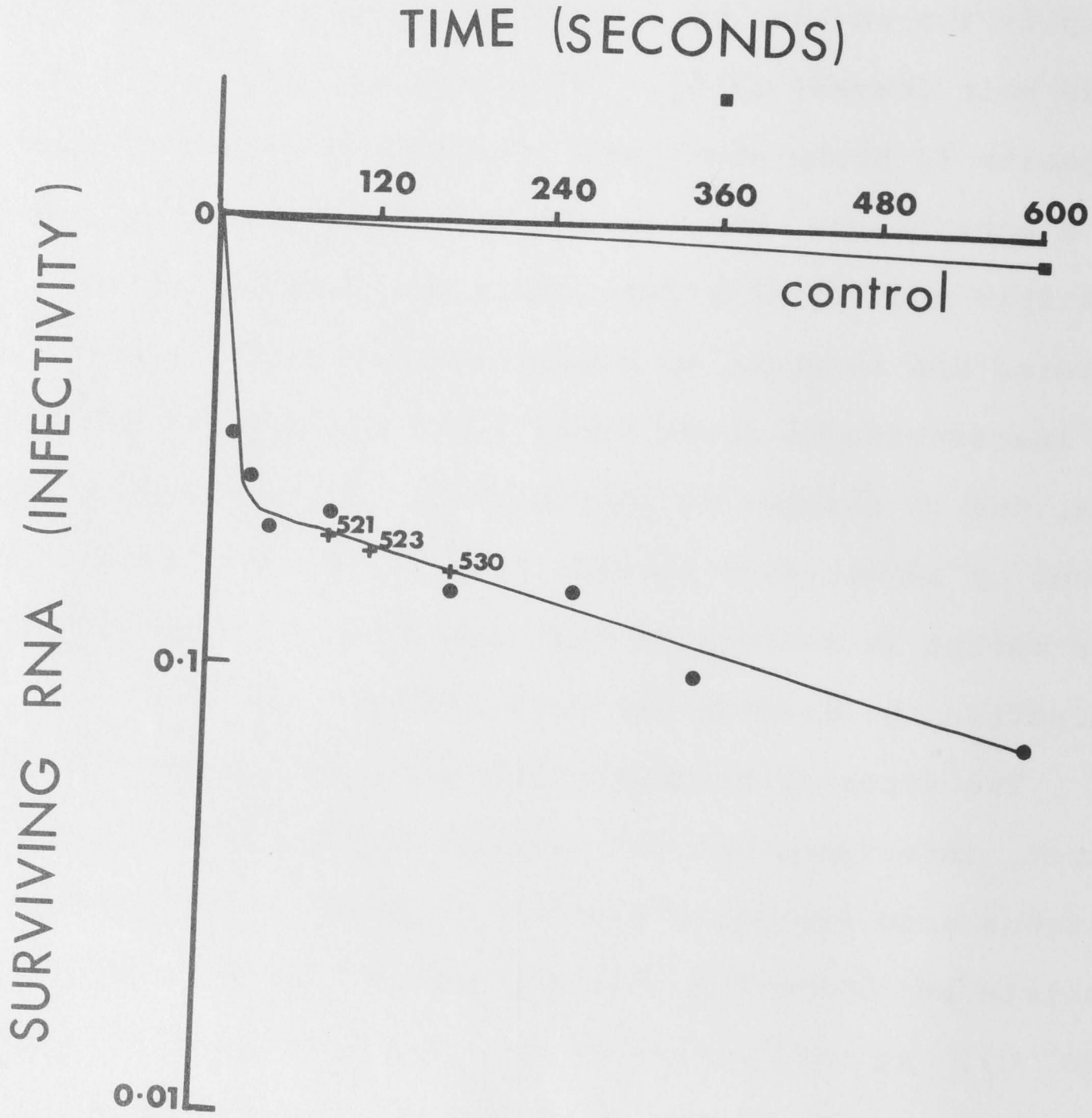


Figure 5: Kinetics of inactivation of ts^+ poliovirus infectious RNA by nitrous acid. Equal volumes of infectious RNA and 2 M nitrous acid were mixed and allowed to act at room temperature (20^o C) for varying times. The reaction was stopped by diluting in ID and surviving infectious RNA was determined by the agar-cell suspension assay. Mutants obtained under different conditions of exposure to the nitrous acid are indicated (+).

The kinetics of inactivation obtained from a single experiment are shown in Figure 5. A biphasic type of inactivation is evident from the curve with a rapid initial rate of inactivation and a flattening, which occurs after about one minute. The same type of inactivation was obtained in several other experiments. Carp and Koprowski (1962) noted a linear single-hit type of inactivation of type III poliovirus RNA with nitrous acid but, as their initial samples were not taken until after twenty minutes of incubation, it is possible they may not have been able to detect the initial rapid loss in infectivity.

The reasons for the relative resistance of poliovirus RNA to further inactivation with nitrous acid after one minute are not clear. Possibly the initial rapid drop in infectivity occurs as a result of lethal interactions between nitrous acid and poliovirus RNA with the remaining fraction being shielded from further attack due to aggregation or RNA secondary structures. A similar relative resistance to inactivation was noted in the venom phosphodiesterase experiment. A number of similar experiments were carried out and superimposed upon Figure 5 are the mutants obtained under various conditions of exposure to nitrous acid.

In the second procedure, poliovirus infectious RNA was treated with nitrous acid for intervals of up to five minutes. The reaction was stopped by dilution as before and the remaining RNA was used to infect U cell monolayer cultures in milk dilution bottles. Three days at 37° C were allowed for virus growth and, after freezing and thawing, the multiple cycle progeny was tested for the presence of ts mutants.

Because of the relative ease in plating whole virus compared with infectious RNA, the latter is a more convenient procedure for selecting mutant plaques. However, a theoretical disadvantage of the use of multiple cycle progeny arises from the possibility of selecting 'sisters' of the same mutant in the infectious progeny. By directly plating mutagen-treated infectious RNA this problem does not arise.

A total of three ts mutants was obtained by directly plating infectious RNA and six mutants were obtained from multiple cycle infectious RNA progeny. With both procedures an isolation rate of around 1 per cent of the total progeny was attained. The best of these mutants was prepared for use in recombination tests which are described in the following section.

Table 3

Characters of *ts* mutants obtained by snake venom phosphodiesterase and nitrous acid [a].

Mutagen	Treatment	Mutant No.	e.o.p. 39.5° [b]	Revertant Content [c]
Snake venom phospho-diesterase	Direct RNA Plating	701 [d]	$1.0\text{-}6.2\times10^{-4}$	6.2×10^{-4}
		702 [d]	1.0×10^{-4}	1.0×10^{-4}
		703	3.0×10^{-4}	$<1.0\times10^{-5}$
		704	5.0×10^{-2}	$<1.0\times10^{-5}$
		705	1.2×10^{-2}	1.2×10^{-2}
		706	3.0×10^{-3}	$<1.0\times10^{-5}$
		707	2.5×10^{-3}	$<1.0\times10^{-5}$
Nitrous acid	Direct RNA Plating	521	Variable: 3.0×10^{-3}-3.0×10^{-5}	$<1.0\times10^{-5}$
		523 [d]	1.0×10^{-4}	5.5×10^{-4}
		530	1.0×10^{-4}	$<1.0\times10^{-5}$
Nitrous acid	Multiple Cycle RNA Progeny	522	$<1.0\times10^{-5}$	$<1.0\times10^{-5}$
		524	2.7×10^{-5}	$<1.0\times10^{-5}$
		525	6.5×10^{-5}	$<1.0\times10^{-5}$
		526	6.0×10^{-5}	$<1.0\times10^{-5}$
		527	2.1×10^{-3}	8.0×10^{-5}
		528	1.0×10^{-4}	$<1.0\times10^{-5}$

a. Mutagen treatment and isolation procedures are described in text or under Materials and Methods.

b. Taken as the ratio of the total plaque count at 39.5° C to the count at 37° C.

c. Taken as the ratio of revertant (*ts*$^+$) plaques produced at 39.5° C to the total count obtained at 37° C for all virus.

d. Mutants actually examined in recombination tests.

1.3 Attempts to classify some mutant isolates by recombination analysis

Table 3 is a list of mutants that were isolated according to the procedures described in sections 1.1 and 1.2. The various mutagen treatments used are indicated in the table, together with the growth and reversion characteristics of each mutant at restrictive temperatures. For several mutants the growth at 38.5^{o} C compared with that at 37.2^{o} (e.o.p. $_{39.5^{o}}$) was less than 1.0×10^{-4} and stocks of some of these were prepared for use in standard recombination tests, which are described in Materials and Methods.

Most mutants isolated after treatment with nitrous acid, had e.o.p. $_{39.5^{o}}$ values lower than those mutants obtained from infectious RNA after treatment with venom phosphodiesterase. Furthermore, in the majority of nitrous acid mutants, the plaques appearing at 39.5^{o} were small with 'leak' rather than revertant characteristics. By analogy with other poliovirus ts mutants such mutants could have multiple genetic lesions (Cooper, 1968).

Mutants obtained after treatment with venom phosphodiesterase diesterase exhibited a range in e.o.p. $_{39.5^{o}}$ values from 1.2×10^{-2} to 1.0×10^{-4}. Plaques appearing at 39.5^{o} C were quite frequently revertant (ts^{+}) in character, which argues against the genetic defect in

TABLE 4

RESULTS OF THE CROSSES 28g x 3, 28g x 701 AND 28g x 702[a]

Cross	Virus Assays[b]												Percentage in yield of		Recombination frequencies[c]	
	39.5°, 10 µgm/ml.			39.5°, 200 µgm/ml.			37°, 10 µgm/ml.			37°, 200 µgm/ml.						
	10^{-2}	$1/3x10^{-2}$	PFU/ml. x 10^{5}	10^{-2}	$1/3x10^{-2}$	PFU/ml. x 10^{5}	10^{-5}	$1/3x10^{-5}$	PFU/ml. x 10^{5}	10^{-5}	$1/3x10^{-5}$	PFU/ml. x 10^{5}	ts+	ts+ g	ts+	ts+ g
3 self	6,2	0,1	0.03	0,0	0,0	-	54,51	18,19	534	0,0	0,0	-	0.006	<0.001	-	-
28 g self	11,6	3,3	0.09	8,10	3,3	0.09	49,40	12,13	429	51,42	14,12	447	0.021	0.021	-	-
701 self	24,18	9,6	0.21	0,0	0,0	-	31,39	10,10	338	0,0	0,0	-	0.062	<0.001	-	-
702 self	31,25	7,6	0.26	0,0	0,0	-	91,81	22,20	804	0,0	0,0	-	0.032	<0.001	-	-
ts+ g self[d]	43,50	13,17	462	65,32	10,12	447	49,48	12,10	447	34,50	24,14	459	100	100	-	-
ts+ self[d]	71,94	39,-	914	0,0	0,0	-	79,81	32,27	823	0,0	0,0	-	100	<1	-	-
28g x 3	SC,SC[e]	60,69	1.94	97,118	39,39	1.10	58,48	16,16	519	19,23	8,14	241	0.374	0.212[f]	0.347	0.191
28g x 3	SC,SC	48,57	1.58	122,89	40,37	1.08	42,31	14,9	361	19,19	5,10	199	0.438	0.299	0.411	0.278
28g x 701	62,56	17,24	0.60	36,38	14,17	0.39	50,34	17,9	414	25,21	9,10	244	0.145	0.094	0.062	0.073
28g x 701	75,73	24,22	0.73	44,47	10,-	0.42	58,55	15,14	534	25,25	11,8	259	0.137	0.079	0.054	0.058
28g x 702	89,84	33,22	0.86	6,-	0,2	0.05	66,70	18,20	654	27,28	5,8	256	0.131	0.008	0.078	0
28g x 702	65,65	25,27	0.68	7,7	-,1	0.06	65,75	22,22	692	33,16	14,5	256	0.098	0.009	0.045	0

(a) The procedure was the standard recombination test described in Materials and Methods.

(b) Plates and bottles were incubated for three days at 37° and 39.5° C respectively. One tenth of a millilitre of the dilutions indicated was added to each assay container.

(c) After correction for spontaneous reversion.

(d) All dilutions of ts+ and ts+g plated were 10^{-5} and $1/3x10^{-5}$.

(e) S.C. = semi confluent.

(f) No correction for guanidine enhancement was made.

the particular mutants concerned being caused by 3′ terminal deletions. It seems improbable that ordered regeneration of a terminal segment, several nucleotides in length, could occur at the reversion rates indicated for these mutants in Table 3.

Two venom phosphodiesterase mutants, ts-701 and ts-702, were examined in duplicate for their ability to recombine with ts-28g, using the standard recombination test described in Materials and Methods. Standard crosses of ts-28g x ts-3 were carried out in parallel, using appropriate controls, and assays of the progeny from each test were conducted at 37.2° C and 39.5° C in the presence of high and low guanidine concentrations. The complete experimental results from these crosses are set down in Table 4 and the summarised recombination frequencies are presented in Figure 6. Also included in Figure 6 are the recombination frequencies from the cross 28g x 523 which was carried out on another occasion, using a different set of controls. The recombination frequencies in the latter cross are standardised by means of the reference cross 28g x 3, which was used in both experiments, but individual data here are not presented.

From Figure 6 the defect in ts-701 appears to be located some 0.06 map distance units to the right of the

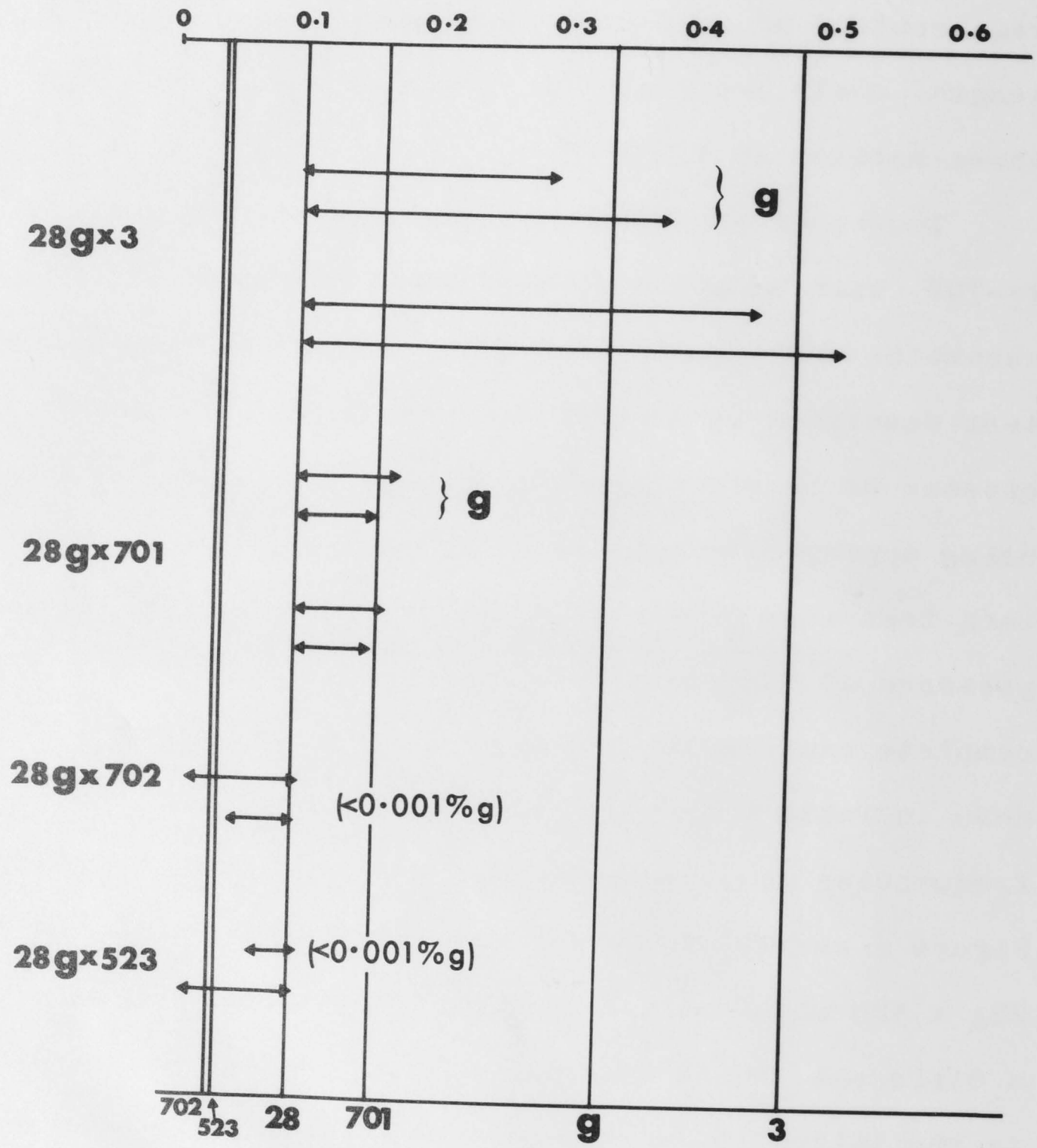


Figure 6: Summary of data from recombination tests. Recombination frequencies from replicate crosses of 28g x 701 and 28g x 702 in Table 4 are arranged in relation to the standard cross 28g x 3, which was carried out at the same time. Also included are data for the cross 28g x 523 obtained in another experiment. Frequencies from the latter cross are standardised in relation to the other tests by reference to the standard cross 28g x 3. The ts defect in ts-28g is arbitrarily fixed at 0.1 map distance units, as in Figure 1.

ts defect in ts-28g and this is supported by the similar values obtained for both ts^+ and ts^+g recombinants. Mutant ts-702, which produced no ts^+g recombinants in crosses with ts-28g, appears to possess a ts defect about 0.06 map distance units to the left of that in ts-28g. Other ts defects may exist to the right and left of those detectable in ts-701 and ts-702 respectively, but further tests of additive recombination frequencies are necessary to demonstrate this, using mutants with known single defects to right of the defect in ts-701 and to the left of that in ts-702. Mutant ts-523 appears to have at least one ts defect approximately 0.05 units to the left of the ts defect in ts-28g.

As mentioned earlier in the Introduction, mutants that are defective in their capacity to allow the synthesis of viral RNA fall into two functional categories, which are represented by ts-20 and ts-28 g. Mutants ts-702 and -523 have defects which appear to lie close to that of ts-20 (see Figure 1), which is defective in its capacity to allow the synthesis of both single and double-stranded RNA under restrictive conditions. Mutants with loci to the right and to the left of defect in ts-701 are defective in their capacity to allow synthesis of single-stranded, but not double-stranded RNA under similar

conditions (Cooper, et al., 1969). Such properties have been cited by Cooper et al. as evidence for the existence of two polymerase enzymes, one concerned with double-stranded template formation and the other with the synthesis of nascent progeny RNA. The actual *in vitro* separation of foot-and-mouth disease viral polymerase into two functional components (Arlinghaus and Polatnick, 1969) tends to confirm the genetic evidence.

If a two-enzyme model is operative, then it seems highly improbable that the defect in *ts*-701 is produced as a result of a 3′-terminal deletion, for such a mutant would be lacking in that part of the genome which specified the first polymerase enzyme required for the synthesis of viral RNA. As mentioned above, both *ts*-701 and *ts*-702 have reversion characteristics which also suggest they are not deletion mutants. Thus *ts*-701 and -702 may have arisen by spontaneous mutation, in which case they probably possess single defects, or they may have arisen from some hitherto unexplained mutagenic property of snake venom phosphodiesterase. The relatively high total isolation rate for these mutants (1-10 per cent) is difficult to reconcile with spontaneous mutation. It was unfortunate that more nitrous acid mutants were not examined in recombination tests for it would be interesting to know whether all these

mutants have defects in the same region of the genome as for ts-523.

Summary

Additional ts mutants of poliovirus were obtained after treatment of poliovirus infectious RNA with the mutagen nitrous acid or the exonuclease snake venom phosphodiesterase and the kinetics of inactivation of infectious RNA for either reagent was studied. It was hoped to produce deletion mutants at the 3′ terminus of the viral RNA with venom phosphodiesterase.

Three of the best of the mutants were used in recombination tests with the well characterised mutant ts-28g, and each was shown to have at least one defect in the region of the genome concerned with viral RNA synthesis. Reasons are given for believing that the mutants obtained after treatment with snake venom phosphodiesterase do not arise due to exonuclease activity.

EXPERIMENTAL SECTION 2

Polymerase Studies with *ts* Mutants of Poliovirus

Poliovirus RNA synthesis is mediated by a viral coded enzyme referred to in this section as an RNA polymerase, which is induced in susceptible cells in response to infection. The existence of such an enzyme or complex of enzymes (in this section the term 'enzyme' is used collectively to mean either) had been previously inferred from two observations in mengovirus infected L cells:- (a) viral replication may take place in the absence of cellular DNA function and (b) replication can be prevented by the addition to infected cell cultures of puromycin, an inhibitor of protein synthesis (Franklin and Baltimore, 1962). The first *in vitro* demonstration of viral polymerase activity was achieved by Baltimore and Franklin (1963) using mengovirus infected L cells, and Baltimore (1964) described a similar activity in HeLa cells infected with type II poliovirus.

Several *ts* mutants of poliovirus are defective in their capacity to synthesise viral RNA when grown in cells at 39.6^{o} C (Wentworth et al., 1968) and it seemed reasonable to believe that at least some of these mutants owe their defectiveness to mutations in genes that either

Table 5

Effects of varying the components of the reaction mixture upon polymerase activity [a]

Experiment	Variation [b]	Polymerase Activity [c]	Per Cent of Control
A	nil (i)	79.5	100
	nil (ii)	75.6	
	Minus ATP, CTP and UTP	4.1	5
	Boiled enzyme	8.5	11
	Minus ATP generating system	36.4	47
	Plus 10 μg RNA'se [d]	50.6	65
	Plus 1 mg bentonite [d]	97.2	125
B	nil	41.0	100
	Plus 100 μg puromycin [d]	39.9	97
	Plus 100 μg cyclo-heximide [d]	38.3	93

a. Standard mixtures were prepared as described in Materials and Methods using a 6 hour ts+ polymerase preparation. Incubation was for 30 minutes at 35° C.

b. Details of variations in procedure are given in text.

c. Polymerase activity is expressed as μμmoles GTP per mg protein in the enzyme preparation.

d. Added in a volume of 50 μl to complete reaction mixtures.

code for or have functions closely associated with viral RNA polymerases. A comparative study was, therefore, made of the polymerases induced in U-cells in response to infection by a number of ts mutants of poliovirus, in comparison with the parental strain ts^+. These studies are described in the latter part of this section. The first part is concerned with characteristics of the polymerase induced by strain ts^+ in U cells, as a basis for comparison with polymerases induced by ts mutants.

2.1 Studies with the polymerase induced by poliovirus strain ts^+

(a) Effect of varying the components of the polymerase reaction mixture

In an examination of the type of polymerase activity induced in U cells in response to infection by ts^+, the standard reaction mixture described in Materials and Methods was modified in several ways and the effects of such modifications are shown in Table 5. Polymerase activity is measured as incorporation of $[^3H]$ GTP into a TCA insoluble fraction and, from reference to radioactive standards, is expressed as μμmoles of GTP incorporated per milligram of total protein in the preparation. In a separate experiment, the effects of two inhibitors of protein synthesis upon in vitro polymerase activity were studied and these results are also included in the table.

Incubation of all mixtures was for thirty minutes at 35° C.

The results suggest that the enzyme responsible for incorporation is a heteropolymerase, that is an enzyme which requires ribonucleoside triphosphates other than GTP as substrate. Incorporation in mixtures containing GTP alone, was only about 5 per cent of the value obtained when all four ribonucleoside triphosphates were present, and this could reflect homopolymerase activity. Such enzymes have been described from both infected and uninfected cells, which are capable of termally incorporating ATP into pre-existing RNA, particularly 4S RNA (Dalgarno et al., 1966). However, incorporation by GTP alone may constitute non-enzymic 'back-ground' activity, as the heteropolymerase activity of a normal reaction mixture was reduced to a level a little in excess of that in mixtures containing GTP alone, when the polymerase preparation was first boiled for five minutes before using in the mixture. Such a procedure would normally destroy most types of enzyme activity.

Polymerase activity in reaction mixtures containing no added ATP generating system was reduced by about 53 per cent. The reasons for this are not clear, but do not appear to be related to the presence of ATP'ase activity,

as a considerable excess of ATP over that needed for RNA synthesis is present in the complete reaction mixture. In reaction mixtures, maximum levels of GTP incorporated were of the order of 100 μμmoles per mg protein; 50,000 μμmoles of ATP were present.

The addition of 10 μg of ribonuclease to the complete standard mixture caused a reduction of only about 35 per cent in polymerase activity. The same level of ribonuclease under identical conditions of incubation completely degrades single stranded poliovirus RNA (data not presented). Baltimore and Franklin (1963) observed a similar relatively low level of inhibition by ribonuclease, using polymerase preparations from cells infected with mengovirus.

The addition 1 mg of bentonite to reaction mixtures stimulated polymerase activity by about 25 per cent, due probably to its action upon ribonuclease and other degradative enzymes upon newly synthesised RNA or template RNA (Brownhill, Jones and Stacey, 1959). Evidence for this was shown in a separate experiment (data not presented) in which the reduction in polymerase activity caused by adding the addition of 10 μg of ribonuclease was completely reversed by the simultaneous addition of 1 mg of bentonite to a reaction mixture.

Puromycin and cycloheximide, both potent inhibitors of protein synthesis in animal cells, had only slight inhibitory effects upon *in vitro* viral polymerase activity. This finding is in agreement with that of Eggers, Baltimore and Tamm (1963) for the polymerase of mengovirus. The results from these experiments indicate that the type of activity measured in complete reaction mixtures is similar to that reported for the polymerases of other RNA viruses (Baltimore and Franklin, 1963; Horton, Liu, Martin and Work, 1966; Martin and Sonnabend, 1967; Scholtissek and Rott, 1969).

(b) *The kinetics of in vitro polymerase activity*

The time course of *in vitro* polymerase activity was studied for individual preparations of viral polymerase by preparing a series of standard polymerase reaction mixtures and incubating for various times. The reaction was stopped after incubation by adding 0.5 ml of 0.1 M sodium pyrophosphate to each tube and placing the tube in an ice bath. After precipitation and washing with 10 per cent TCA, as described in Materials and Methods, polymerase activity was determined from the radioactivity of each sample.

The results of two separate experiments are shown in Figure 8. In one experiment (open symbols) the kinetics

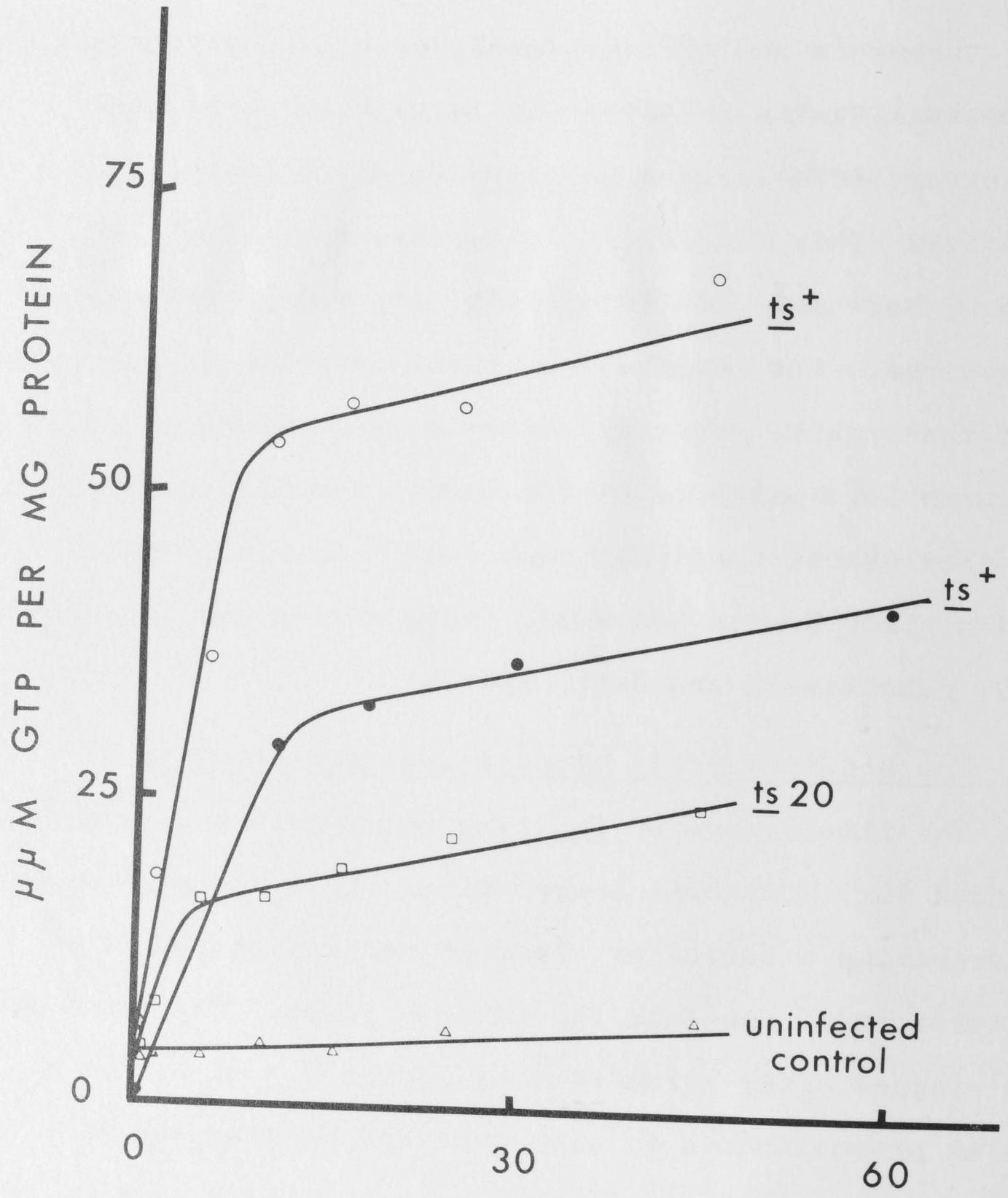


Figure 7: The kinetics of *in vitro* polymerase activity. Standard reaction mixtures containing 6 hour ts$^+$ or ts-20 polymerase preparations from infected cells, or an extract from uninfected cells, were incubated for varying times as described in the text. The reaction was stopped by the addition of 0.5 ml of 0.1 M sodium pyrophosphate. The results for two experiments are shown. In one (open symbols) an incubation temperature of 37^o C and an ATP generating system consisting of creatine phosphokinase/creatine phosphate was used; in the other (closed symbols) the temperature was 35^o C and the ATP generating system pyruvate kinase/2-phosphoenol pyruvate.

of incorporation at 37° C are shown, using polymerases prepared from cells infected with either ts$^+$ or ts-20 for six hours at 37° C. Also included in this experiment are the results from a parallel series containing an uninfected cell extract prepared in the same manner as the polymerase preparations. The ATP generating system consisted of creatine phosphokinase/creatine phosphate (6E.U./6μmoles). In a second experiment (closed symbols) the kinetics of a different preparation of ts$^+$ polymerase were studied using an incubation temperature of 35° C and 2-phosphoenol pyruvate/pyruvate kinase (6 E.U./6μmoles) as the ATP generating system.

The results from Figure 7 suggest that the kinetics of polymerase activity for all preparations are very similar, with near-maximum values for incorporation being attained after 15 minutes of incubation and further increases in activity after this time being relatively small. In the first experiment, the final level of activity for the polymerase of ts-20 appeared to be significantly lower than that of ts$^+$. This finding has been noted elsewhere (Experimental Section 2.3) and appears to be a feature of the production of polymerase by this mutant at temperatures between 33 and 39.5° C.

Polymerase activity in the uninfected control preparations

showed only a slight increase with time of incubation and remained at levels that corresponded to the zero time values for the polymerases of ts$^+$ and ts-20 in the first experiment. Similar kinetics of incorporation have been noted, without exception, for several other animal virus-induced RNA polymerases (Baltimore and Franklin, 1963; Horton et al., 1966; Martin and Sonnabend, 1967; Plagemann and Swim, 1968; Scholtissek and Rott, 1969).

Further experiments (data not presented) revealed that 35^o C was the most suitable incubation temperature for ts$^+$ polymerase. At this temperature, there appeared to be little difference in activity when either ATP generating system was used, and, for reasons of availability, 2-phosphoenol pyruvate/pyruvate kinase, at the concentration described above, was used throughout the remainder of the experimental work. On the basis of the results in this section, and unless otherwise stated, an incubation time of 30 minutes and a temperature of 35^o C was used for determining the activity of all polymerase preparations.

(c) A consideration of the errors present in the assay system for ts$^+$ polymerase

In this experiment three batches of polymerase were prepared and were assayed in triplicate on three separate

Table 6

Results of assays of different polymerase preparations conducted at different times [a]

	Preparation 1	Preparation 2	Preparation 3	Row Means
Week 1	20.4 [b] 25.6 24.5	26.0 23.5 36.5	15.8 23.2 25.4	24.5
Week 2	16.7 18.0 13.4	23.2 25.0 22.8	26.2 27.0 21.8	21.6
Week 3	25.8 22.8 20.7	38.2 31.5 31.1	29.2 21.0 25.5	27.3
Column Means	20.9	28.6	23.9	

a. Details for this experiment are given in the text.

b. Results of each assay are expressed as μμM GTP per mg protein in the enzyme preparation.

occasions. A single large batch of U cells (approximately 1.0×10^9 cells) was infected with $\underline{ts}^+$ at a multiplicity of 3-5 p.f.u. per cell and in a final volume of 20 ml, for two hours at 4^o C. One third of the infected suspension was then placed in each of three spinner flasks containing 200 ml of Spinner Medium (see Materials and Methods), which was pre-warmed to 37.2 C in a waterbath. Incubation of the spinner cultures at 37.2^o was continued for 6 hours when they were chilled, and separate preparations of polymerase were made from the cells of each flask, as described in Materials and Methods. Each polymerase preparation was then divided into three separate 1 ml lots, which were stored at -15^o C.

Assays of a single batch of each preparation were carried out on the following day and then at two successive weekly intervals, using the standard reaction mixture outlined in Materials and Methods and an incubation time of 30 minutes at 35^o C. Estimates of the protein content of each polymerase preparation were also made and the summarised results of all assays are presented in Table 6.

The significance of errors arising from the use of different polymerase preparations and those due to the assays being carried out on separate occasions (Factors

Table 7

Analysis of variance of the data in Table 6

Source of Variation	Degrees of Freedom	Mean Square	F	Significance
Between Weeks (W)	2	74.28	$\frac{74.28}{18.24} = 4.07$	0.05>P>0.025*
Between Preparations (P)	2	137.95	$\frac{137.95}{18.24} = 7.56$	0.01>P>0.001**
WP Interaction	4	33.01	$\frac{33.01}{14.96} = 2.21$	P>0.1
Residual Variance	18	14.96		
Total	26			

P and W, respectively) was assessed, statistically, by applying a two-factor analysis of variance to the data in Table 6. A summary of the components of variance is presented in Table 7.

From the analysis, the variance due to interaction WP, when tested against that of the residual variance, was found to be not significant ($P < 0.1$) and this allowed for a revised estimate of the residual variance by pooling the sums of squares of the old residual with those of interaction WF, and dividing by the combined number of degrees of freedom. Each of the variances of the main-effects W and P were then shown to be significantly greater than the new residual variance. Thus, errors in assays of replicate polymerase preparations when carried out at the one time, and those arising when assays of the same preparation are made at weekly intervals are significantly greater than errors in replicate assays of a single preparation when carried out at the one time.

These findings may not apply for polymerase samples with activities of 70-100 μμ M GTP per mg of protein, which are frequently obtained from $\underline{ts}^{+}$ infected cells. However, because of the numbers of cells required to obtain as many samples with this level of activity, a similar analysis was not carried out. The variability

that occurs between samples prepared in replicate spinner flasks may arise from any of the numerous steps involved in preparing the polymerase enzyme. Such errors must be taken into account in the interpretation of some results in Experimental Section 2.2, where the nature of certain experiments demands a comparison between polymerases prepared from cells in different spinner flasks.

From Table 6, the mean of all values from each time of assay suggest that there is no significant loss in activity due to storage at -15° C. Other findings to be described in Experimental Section 2.2 also suggest that preparations of ts$^+$ polymerase are relatively stable.

(d) The relationship between polymerase activity and relative concentration

For most enzymes, a linear relationship exists between activity (maximum velocity) and relative concentration over much of the range of enzyme concentration (Dixon and Webb, 1958). Where an attempt is made to compare levels of enzyme activity between various preparations, as with the polymerase assays in this section, a demonstration of linearity is necessary as a criterion of validity for the particular assay system.

In an attempt to demonstrate such a relationship in the polymerase assay system described above, a closely

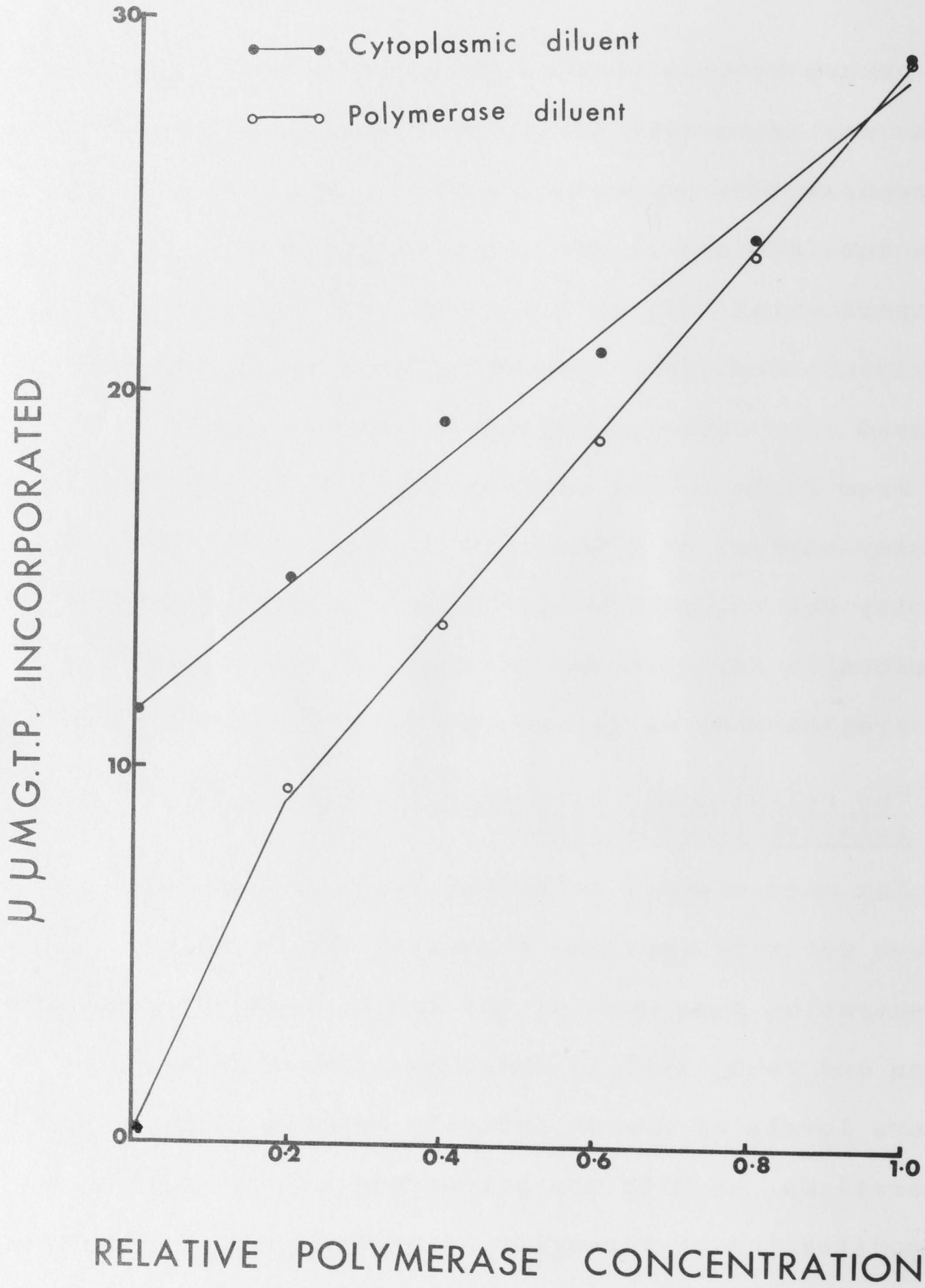


Figure 8: The relationship between polymerase activity and relative concentration. Dilutions of a $\underline{ts}^{+}$ polymerase preparation were made (a) in a cytoplasmic extract obtained from uninfected cells and adjusted with Polymerase Diluent to the same protein concentration (1.21 mg per ml) as was present in the polymerase preparation, and (b) Polymerase Diluent. Standard reaction mixtures containing the diluted polymerase were then prepared and activities determined in the usual manner.

spaced series of dilutions of a preparation of ts$^+$ polymerase was made. The diluents used were (a) Polymerase Diluent (see Materials and Methods), and (b) an uninfected cytoplasmic extract, which was prepared from the same batch of cells and adjusted to the same protein concentration as for the polymerase (1.21 mg/ml) with Polymerase Diluent. Reaction mixtures containing the diluted polymerase were then prepared and enzyme activity was determined from the TCA insoluble radioactivity of each mixture, after incubation at 35° C for 30 minutes. The results obtained are presented in Figure 8, which shows that a linear relationship exists between enzyme activity and relative concentration of enzyme in both diluents.

In neither case do the lines obtained extrapolate to zero activity with the ordinate and this could be due to either non-enzymic incorporation of GTP, or to some enzymic activity, which catalyses the addition of GTP to pre-existing cellular RNA (see Experimental Section 2.1(a)). Such activity can be seen at low polymerase concentrations, where there is a considerable difference in incorporation between preparations that are diluted in Polymerase Diluent and uninfected cytoplasm. This

difference is probably due to the 'background' in corporation by uninfected cell extracts.

Thus, in the preparation examined, a linear relationship exists between polymerase activity and relative enzyme concentration. Similar findings were also noted in a preparation of ts-28 polymerase with a protein concentration of 1.78 mg per ml and an activity of 16 μμM GTP per mg protein. A linear relationship has been demonstrated between activity and relative concentration for the polymerase of encephalomyocarditis virus, (EMC; Horton, et al., 1966) and Semliki Forest virus (Martin and Sonnabend, 1967).

(e) The time of synthesis of polymerase, infectious RNA and complete virus in U cells infected with ts^+

In this experiment an attempt was made to correlate the synthesis of polymerase, infectious RNA and complete infectious virus in infected U cells. About 6.0×10^8 cells were infected with ts^+ at a multiplicity of 3-5 p.f.u. per cell in a final volume adjusted to 20 ml with PBS. The suspension was rocked at 4° C for two hours to allow virus to adsorb, before transferring the suspension to a large spinner flask containing 180 ml of Spinner Medium (see Materials and Methods), prewarmed to 37.2° C in a waterbath. A second flask contained an uninfected cell suspension of 2.0×10^8 cells in 60 ml.

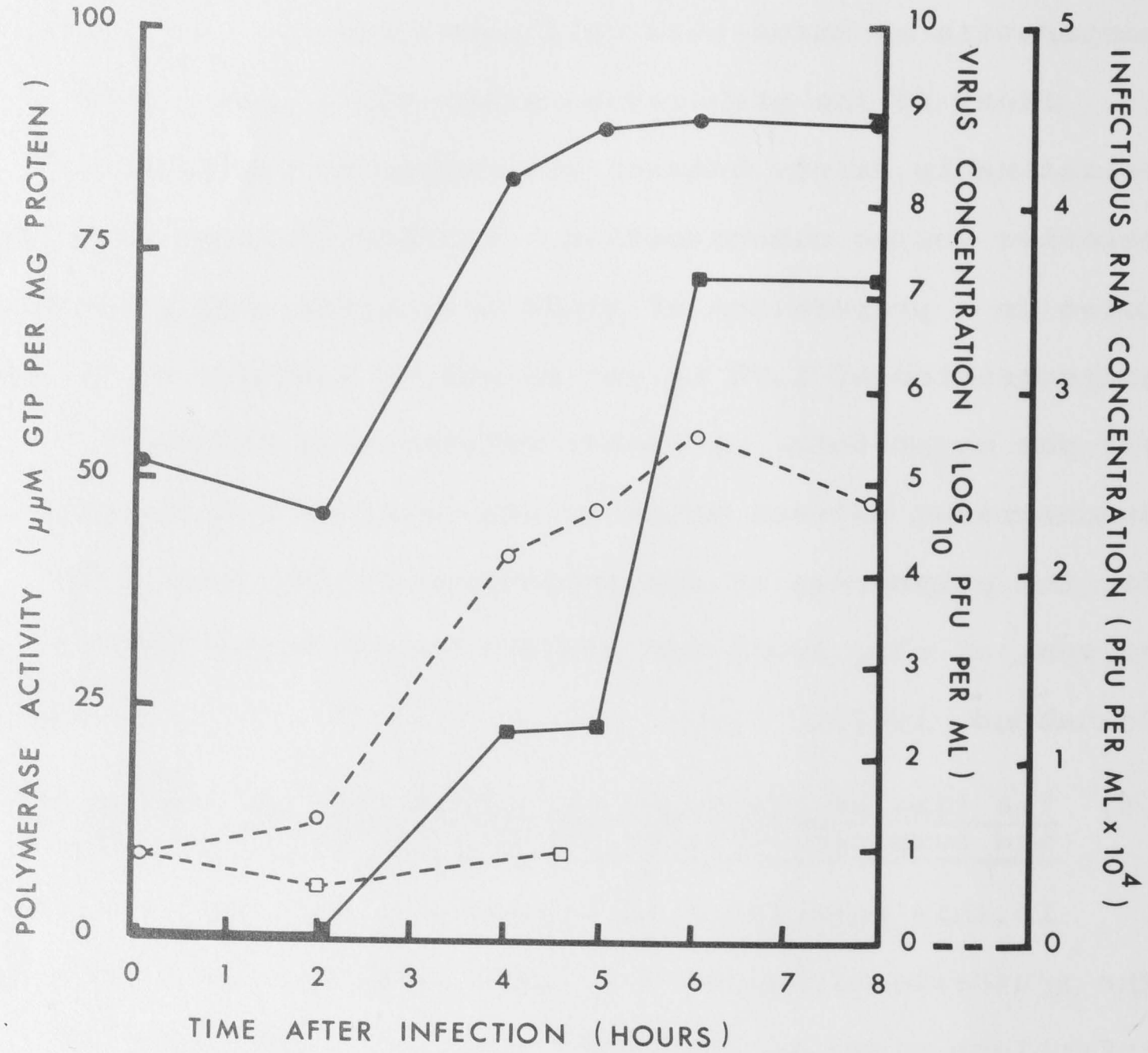


Figure 9: The time course of synthesis of polymerase, infectious RNA and complete virus in U cells infected with ts$^+$. Suspensions of infected and uninfected U cells were grown at 37.2° C in spinner culture and samples taken after varying times. Polymerase activity in cells and the virus and infectious RNA present in whole infected suspensions were determined according to standard procedures.

——●—— = complete virus, ——■—— = infectious RNA,

----○---- = polymerase activity in infected cells,

----□---- = polymerase activity in uninfected cells.

Incubation of the spinner cultures at 37.2° C was continued for several hours and 30 ml samples of each cell suspension were taken at various intervals. Twenty five ml of each suspension were used to prepare polymerase assay samples while the remainder, in the case of the virus infected suspensions, was used for the assay of virus and infectious RNA. Assays of each were carried out as described in Materials and Methods and the results of the experiment are presented in Figure 9.

The results show that maximum levels of polymerase, infectious RNA and complete virus are reached after 6 hours of infection at 37.2° C. By comparison the level of polymerase activity in the uninfected control sample remained fairly constant over the first 4.5 hours. Maximum levels of polymerase were obtained at 6 hours by Baltimore and Franklin (1963) in mengovirus infected L cells, and at 5.5 hours for EMC virus polymerase by Horton, Liu, Dalgarno, Martin and Work (1964). Progeny virus and infectious RNA were first detectable between 2 and 4 hours after infection. According to Cooper (1964 b) the eclipse period for poliovirus is 2.6 hours, and progeny infectious RNA can first be detected at 2.0 hours.

(f) The RNA species produced in vitro by reaction mixtures of ts+ polymerase

This section describes the examination by sedimentation analysis in sucrose gradients of RNA produced by ts+ polymerase reaction mixtures.

Three separate reaction mixtures (A, B and C) were prepared using the same 6 hour ts+ polymerase preparation and the more concentrated reaction mixture for gradient analysis (see Materials and Methods). The isotope used was [^{3}H] ATP (50 mμ M; 1000 c/M) and 1 mg of bentonite was also included because of the enhancement in total activity it confers (Table 5).

Reaction mixture A was incubated for 10 minutes and B and C for 30 minutes at 35° C and RNA was prepared by extraction with phenol/SDS at 60° C, as described in Materials and Methods. To each extract was added 0.2 ml of a preparation of ^{14}C U cell ribosomal marker RNA, and extract C was incubated with 10 μg of ribonuclease for 30 minutes at 25° C.

All three extracts were then centrifuged for 270 minutes at 7° C through pre-formed 15-30 per cent sucrose gradients at 36,500 r.p.m., using a Spinco SW-39 rotor. Fractions from each gradient were collected and one half of those from A and B were incubated with 1 μg of ribonuclease at 25° C for 30 minutes. All fractions were

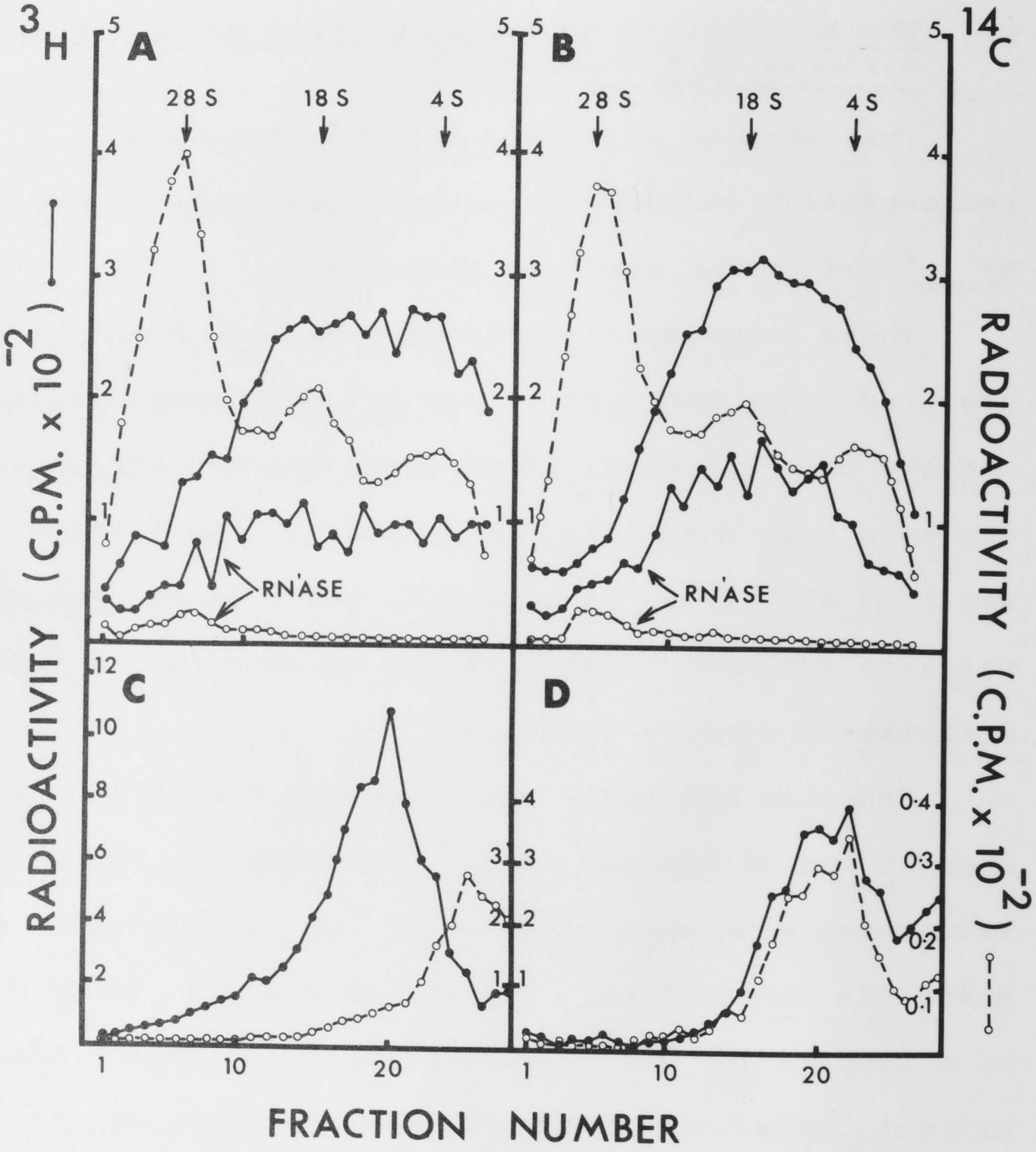


Figure 10: Sucrose-gradient analysis of the RNA product of a ts+ polymerase preparation. Standard reaction mixtures containing bentonite were incubated for 10 minutes (mixture A) or 30 minutes (mixtures B, C and D) and [^{3}H] UTP RNA extracted with phenol/SDS at 60° C. [^{14}C] ribosomal RNA was added to extracts A, B and C and [^{14}C] Replicative Form marker to D. Extracts of C and D were treated with 10µg of ribonuclease for 30 minutes at 25° C and all preparations then centrifuged through 15-30 per cent sucrose gradients for 4.5 hours at 36,500 r.p.m.. Half the fractions from gradients A and B were treated with 1µg of ribonuclease for 30 minutes at 25° C. The distribution of acid-insoluble radioactivity for all gradients is shown.

then placed on paper strips, which were acid washed as described in Materials and Methods, and the radioactivity present on each determined using a liquid scintillation counter.

The results for all three gradients are shown in Figure 10 (A-C). In an additional experiment (D), RNA from a 30 minute reaction product was examined after ribonuclease treatment, as in reaction mixture C, but, in place of ribosomal RNA, 0.2 ml of ^{14}C Replicative Form RNA from poliovirus infected cells (see Materials and Methods) was used as the marker. From Figure 10 a broad distribution of [^{3}H] RNA for the 10 and 30 minute reaction products can be seen in gradients A and B. About half the RNA was resistant to the action of ribonuclease which suggests that a complex of single- and double-stranded RNA is present, such as is found in the Replicative Intermediate obtained from in vivo pulse-labelling experiments (Baltimore and Girard, 1966). RNA from the 10 minute reaction mixture is distributed more broadly to contain more low-molecular weight material than can be seen in B, where RNA from the 30 minute reaction mixture was examined. This could be related to differences in the length of nascent single-stranded progeny in the complex which may vary according to the length of the incubation

time. Similar RNA profiles were obtained from polymerases prepared from cells 3.0 and 4.5 hours after infection, but no evidence for a separate peak of 35s viral RNA could be found.

The peak of ribonuclease-resistant radioactivity could be sharpened considerably if the product was treated with ribonuclease before centrifuging (gradient C), and, in gradient D, the ribonuclease-resistant component is shown to have a similar sedimentation coefficient to the ribonuclease-resistant Replicative Form.

The results obtained in Figure 10 are in conflict with those of Baltimore (1964) for the product of type II poliovirus RNA polymerase, and by Dalgarno et al. (1966) for that of EMC, in which relatively large peaks of single-stranded viral RNA and smaller peaks of Replicative Intermediate were obtained. More recently, however, RNA polymerase preparations of mengovirus (Plagemann and Swim, 1968), foot-and-mouth disease virus (Arlinghaus and Polatnick, 1969) and poliovirus (Girard, 1969) have been shown to produce RNA structures similar to the Replicative Intermediate observed from <u>in vivo</u> pulse-labelling experiments. Here the RNA sedimented as a heterogeneous species in sucrose gradient's as in Figure 10A and B and was partly resistant to ribonuclease. However, the

distribution of radioactivity for the RNA products of these polymerases was more in the region of the 28S rather than the 18S marker, in contrast to that seen in Figure 10. This difference in distribution may be due to a partial breakdown of the Replicative Intermediate during phenol extraction at 60° C to release partly completed single strands from the complex, or to the action of degradative enzymes in the U cell polymerase preparation.

Such a breakdown after extraction at 60° C with phenol/SDS was noted for *in vivo* labelled poliovirus Replicative Intermediate by Bishop and Koch (1969).

(g) Thermal transition properties of the RNA produced by ts^+ polymerase reaction mixtures

The following experiments were carried out in order to study the stability of RNA species produced by ts^+ reaction mixtures at various temperatures. They also served as additional tests of identity between double-stranded RNA synthesised *in vitro* and Replicative Form RNA.

In order to produce large amounts of polymerase RNA, three standard reaction mixtures containing ts^+ polymerase and 1 mg bentonite were prepared using [^{3}H] GTP (1000 c/M; 50 m μM) as the labelled nucleoside triphosphate. After incubation for 30 minutes at 35° C, RNA was prepared from the reaction mixtures by three extractions with

phenol/SDS at 0° C. A lower temperature of extraction was used in order to preserve any Replicative Intermediate which may have been partly dissociated at the higher temperature (60° C) used in earlier studies. RNA from the reaction mixture was pooled and diluted 1:5 in NET buffer and 0.4 ml amounts were stored in glass vials at -70° C.

In examining the thermal transition properties of the RNA, the contents of each vial were thawed at 0° C, and the vial was attached to the bulb of a thermometer and placed for 10 minutes in a thermostatically controlled glycerol bath, maintained at a particular temperature. The vial was then removed and its contents were immediately frozen by plunging into an ethanol/dry ice bath. A range of temperatures was used and, after thawing, 0.2 ml from each vial were placed on to paper strips, such as were used for gradient fractionation (see Materials and Methods), while the remainder were treated with 1 μg of ribonuclease for 30 minutes at 25° C before placing on another series of strips. All papers were acid washed in the usual manner and the radioactivity present was determined using a liquid scintillation counter. The thermal transition properties of a preparation of *in vivo* labelled *ts*$^{+}$ Replicative Form in the same buffer (see

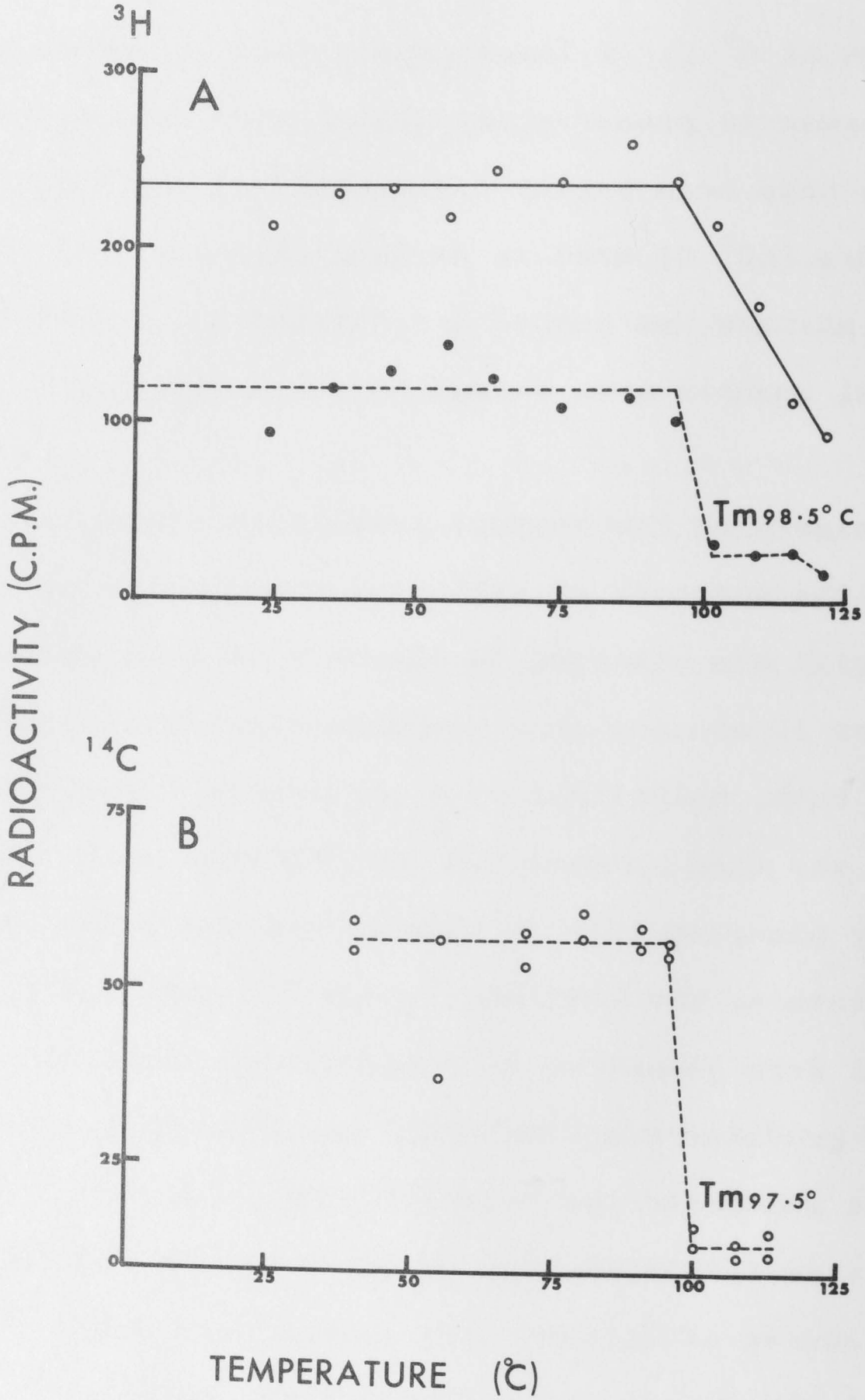


Figure 11: Thermal transition properties of the RNA product of ts$^+$ polymerase preparations. [^{3}H] GTP labelled RNA from three reaction mixtures was obtained by phenol/SDS extraction at 0° C and diluted in NET buffer. Fractions were heated at different temperatures for 10 minutes and then rapidly frozen in an ethanol/dry ice bath. Half of each fraction was treated with 1µg of ribonuclease for 30 minutes at 25° C and the profile of radioactivity at different temperatures for treated (closed symbols) and untreated (open symbols) RNA is shown in A. In B the profile for [^{14}C] labelled Replicative Form RNA after heating and treatment with ribonuclease is shown from a separate experiment.

Materials and Methods) were also studied and the results of both experiments are shown in Figure 11.

In the curves for the polymerase reaction product (A) there is no evidence of RNA dissociation below 97.5° C, the mid-temperature of dissociation (Tm) for the Replicative Form RNA. This result is in contrast to one obtained by Plagemann and Swim (1968) for the reaction product of mengovirus RNA polymerase. They noted the existence of another species of RNA with a lower melting temperature (40-65° C) than that of double stranded RNA, and this material had many properties of the Replicative Intermediate of poliovirus (Baltimore and Girard, 1966). RNA degradative activity may have been responsible for the loss of such an RNA species under the present conditions, and this possibility is examined in the following section.

The melting temperature (Tm) of ribonuclease resistant RNA for <u>in vitro</u> and <u>in vivo</u> labelled preparations was 98.5° and 97.5° C, respectively. Such values are calculated from the mid-temperature between the beginning and end of strand separation of the ribonuclease-resistant fraction, and their near equivalence indicates a close similarity in the duplex structures present in each preparation.

(h) The extent of RNA degradative activity in polymerase reaction mixtures

Relatively low sedimentation values for the RNA complex produced by ts$^+$ polymerase preparations have been noted in comparison with reported values for the poliovirus Replicative Intermediate and the RNA complex produced by other picornavirus polymerases (Section 2.1 (f)). This may be due to some enzymic breakdown of newly synthesised single stranded RNA in the complex, and, in order to test for such activity, the effects upon viral RNA of incubation in a ts$^+$ reaction mixture were studied.

Two normal reaction mixtures, containing a 6 hour ts$^+$ polymerase preparation and 1 mg of bentonite, were incubated with 0.2 ml of ^{14}C labelled RNA from poliovirions. Unlabelled nucleoside triphosphates were used in the mixture and the RNA had been extracted from purified ts$^+$ virions with phenol/SDS at 60^o C. One mixture was incubated for 5 and the other for 30 minutes at 35^o C, and, as a control, a third mixture, containing 0.2 ml of RNA diluent (NET) in place of RNA, was also incubated for 30 minutes. After incubation, all mixtures were extracted with phenol/SDS at 60^o C in the usual manner, and 0.2 ml of the same RNA preparation was added to the control. The three extracts were then centrifuged through pre-formed 15-30 per cent sucrose gradients for 270 minutes at

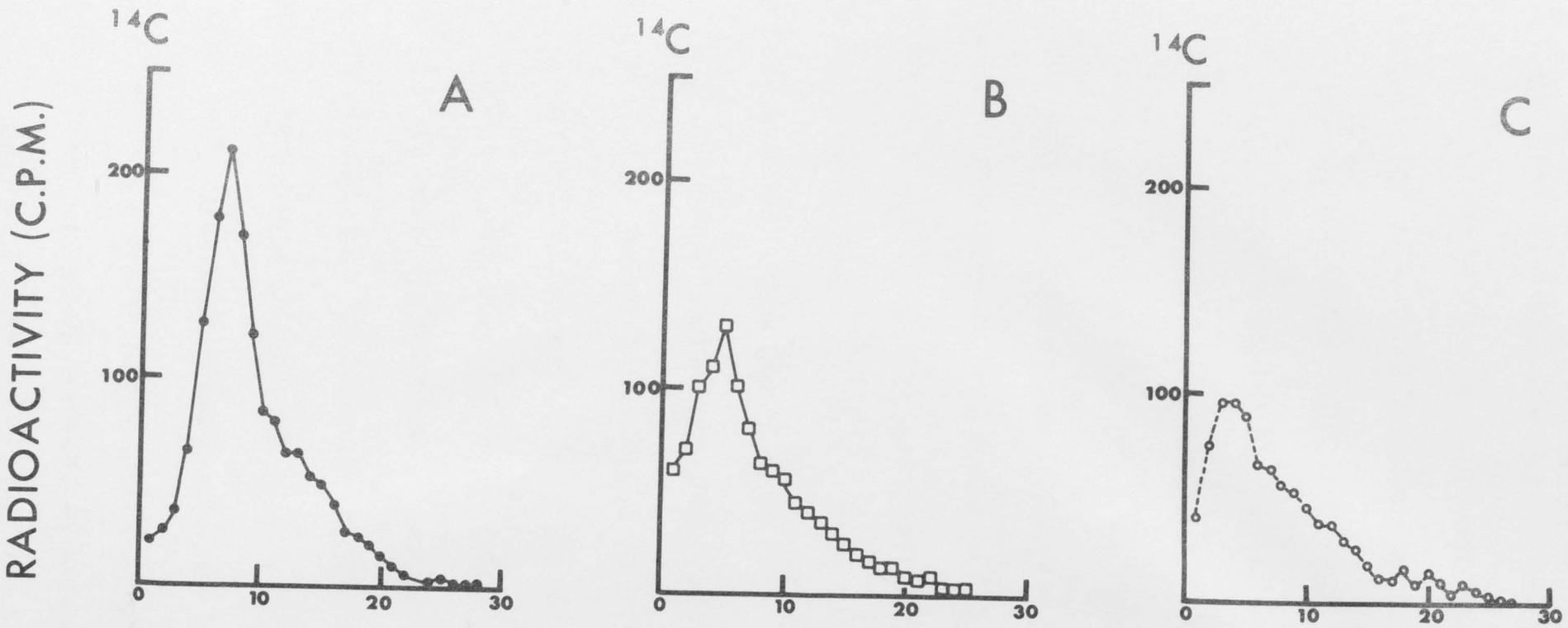


Figure 12: RNA degradative activity in ts^{+} polymerase reaction mixtures. Two standard reaction mixtures B and C, containing bentonite and unlabelled nucleoside triphosphates were incubated at 35° C for 5 and 30 minutes, respectively, in the presence of 0.2 ml of [^{14}C] labelled RNA, prepared from purified poliovirons. RNA from the mixtures was then extracted with phenol/SDS at 60° C and the extracts centrifuged through 15-30 per cent sucrose gradients for 4.5 hours at 36,500 r.p.m. A control mixture (A) contained NET buffer in place of poliovirus RNA but, after incubation for 30 minutes, 0.2 ml of ^{14}C poliovirus RNA was added immediately before centrifugation. The profiles for poliovirus RNA remaining in each mixture are shown.

36,500 r.p.m.. The gradients were fractionated and acid insoluble radioactivity was determined in the usual manner.

The results of the experiment are presented in Figure 12 and suggest that some breakdown in 35S viral RNA occurs under the conditions of incubation used for polymerase reaction mixtures. This degradative activity may be sufficient to account for differences in sedimentation coefficient referred to previously, between the RNA of a polymerase reaction mixture and poliovirus Replicative Intermediate. However, the extent of such a difference is difficult to predict from Figures 10 and 12, as the theoretical capacity of the polymerase reaction mixture to synthesise single stranded RNA is unknown.

RNA degradative activity may be related to different levels of ribonuclease in the cells used to prepare viral RNA polymerases (E.M. Martin, personal communication). Because of this, the RNA profiles obtained from polymerases prepared from ts^{+} infected HeLa and U cells were compared in a single experiment, but no differences were noted (data not presented).

2.2 Studies with ts mutants of poliovirus

(a) The induction of viral polymerase by ts mutants under permissive and restrictive conditions

In experiments described in this section, levels of

Table 8

The induction of viral polymerase by ts mutants at 37.2 and 39.5° C [a]

Experiment	Mutant	Mutant Type [b]	Polymerase Level at 37.2° C	Polymerase Level at 39.5° C
A	ts+	-	21.6 [c]	5.9
	ts-20	RNA mutant	4.7	1.0
	ts-3	protein coat mutant	35.2	11.6
	Uninfected cells	-	0.5	-
B	ts+	-	23.4	7.9
	ts-28	RNA mutant	24.0	5.0
	ts-2	protein coat mutant	9.5	3.9
	Uninfected cells	-	2.2	-
C	ts+	-	18.6	9.1
	ts-20	RNA mutant	7.5	1.4
	ts-104	protein coat mutant	21.6	8.8
	Uninfected cells	-	1.2	-

a. Procedures for the experiment are given in the text and in Materials and Methods.

b. Obtained from data in Table 2.

c. Activities are expressed as μμM GTP per mg of protein in the polymerase preparation.

polymerase in U cells were compared after the growth of several mutants at permissive (37.2° C) and restrictive (39.5° C) temperatures. Suspensions of 2.0 x 10^8 cells were infected in parallel with ts$^+$ or a particular mutant, at a multiplicity of 3-5 p.f.u. per cell and in a final volume of 10 ml. Adsorption was then allowed to take place by rocking the suspension at 4° C for 2 hours, and one half of each infected suspension was then transferred to spinner flasks containing 100 ml of Spinner Medium, which had been pre-warmed in waterbaths maintained at either temperature. Incubation at these temperatures was continued for 6 hours when the suspensions were then chilled, and polymerase preparations were made from the cells of each, as described in Materials and Methods.

The polymerase activity in each preparation and in a 6 hour uninfected control extract were determined and the results of three separate experiments are shown in Table 8. All mutants and ts$^+$ appeared to induce lower levels of polymerase at 39.5° compared with 37.2° C, which is the optimum temperature of virus growth of all mutants and ts$^+$ (P.D. Cooper, personal communication). Mutant ts-20, which at restrictive temperatures is defective in its capacity to allow the *in vivo* synthesis of both double and single-stranded RNA (Cooper, et al., 1969), produced

considerably less polymerase at 37.2° C than did ts$^+$ and the other mutants tested. This finding prompted a study of the optimum temperature for the induction of polymerase by ts-20, which is described in Experimental Section 2.2(c). At 39.5° C the activity of polymerase induced by ts-20 approximated that of the uninfected control preparations.

The remaining mutants, with the exception of ts-2 in a single experiment, induced levels of polymerase that were at least comparable with those of ts$^+$ at permissive and restrictive temperatures. These mutants included ts-28, and some protein coat mutants with single defects occuring in the genetic map to the right of that in ts-20, and these are defective in their capacity to allow synthesis of single-, but not double-stranded RNA, under restrictive conditions (Cooper, et al., 1969).

From these experiments it is clear that the inability of ts mutants to multiply at 39.5° C, in comparison with ts$^+$, is not accompanied by the same relative incapacity to induce polymerase at this temperature i.e. all mutants and ts$^+$ are defective in their capacity to induce polymerase at 39.5° C.

(b) The effects of temperature shift upon the production of polymerase by ts mutants

Cooper et al. (1966), in a study of the effects of temperature shift upon the growth of ts mutants, found that all, with the exception of ts-23, possessed 'late' defects in viral growth. These data are derived from 'step-up' experiments, in which infected cells were transferred from 37 to 39.5° C at different times during the growth cycle.

The effects of 'step-up' procedures upon the induction of polymerase by ts-20, -23 and -28 were studied; all of these are RNA negative mutants (Wentworth et al., 1968). Suspensions of about 5.0×10^8 U cells were infected with each mutant at 4° C for 2 hours and at a multiplicity of 3-5 p.f.u. per cell. About 1.0×10^8 cells from each infected suspension were then transferred to spinner flasks containing 100 ml of Spinner Medium, which had been pre-warmed in 37.2° C waterbaths. Incubation at 37.2° C was continued and, at varying intervals up to 6 hours, individual spinner cultures were transferred to a 39.5° C waterbath. At 6 hours all suspensions were chilled and polymerase preparations were made from the cells of each according to standard procedures. The activity of each preparation was determined and the

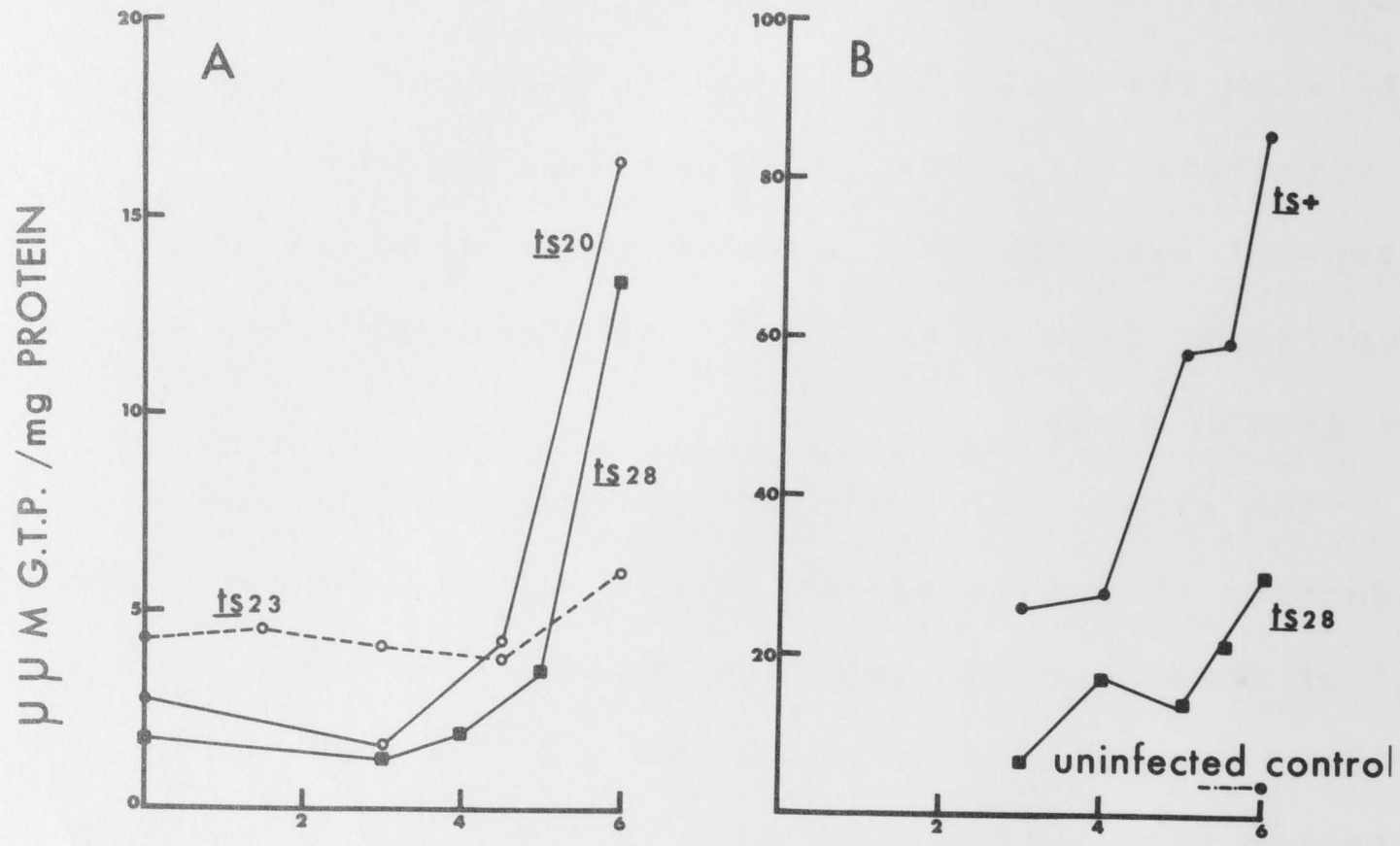


Figure 13: The effects of temperature shift upon the production of polymerase by ts mutants. Suspension of U cells, infected with a particular ts mutant or ts^+, were grown at 37.2° C in spinner culture and, at several intervals up to 6 hours, individual cultures were transferred to 39.5° C. At 6 hours all cultures were chilled and polymerase extracts from the cells prepared and assayed in the usual manner. The results for two experiments (A and B) are shown. In B an uninfected control suspension, incubated for 6 hours at 37.2° C, is also included.

results of two experiments are presented in Figure 13.

From the figure all mutants and ts$^+$ appear to be defective in their capacity to induce polymerase, even when the infected suspensions are transferred to 39.5° C for relatively short intervals before harvesting. In other words, all mutants and ts$^+$ require some 'late' function that is responsible for the induction of polymerase. The result also extends to ts-23, although here the result is in some doubt, because of the very low levels of polymerase inducible at 37.2° C, both in this and in other experiments (unpublished). From experiment B the base level of polymerase induced by ts$^+$ at 39.5° C between 3 and 6 hours appeared higher than that induced by ts-28, although this was not apparent in the 6 hour data for the production of polymerase in Table 8. None of the results for either experiment is consistent with the existence of an 'early' temperature sensitive step in the production of polymerase, which presumably occurs in certain RNA negative mutants of Semliki forest virus with 'early' ts defects in virus growth (Tan, et al., 1969).

(c) The levels of polymerase induced by ts-20 and ts$^+$ at different temperatues of incubation

Following the observation that relatively low levels of polymerase were induced at 37.2° C in 6 hour spinner

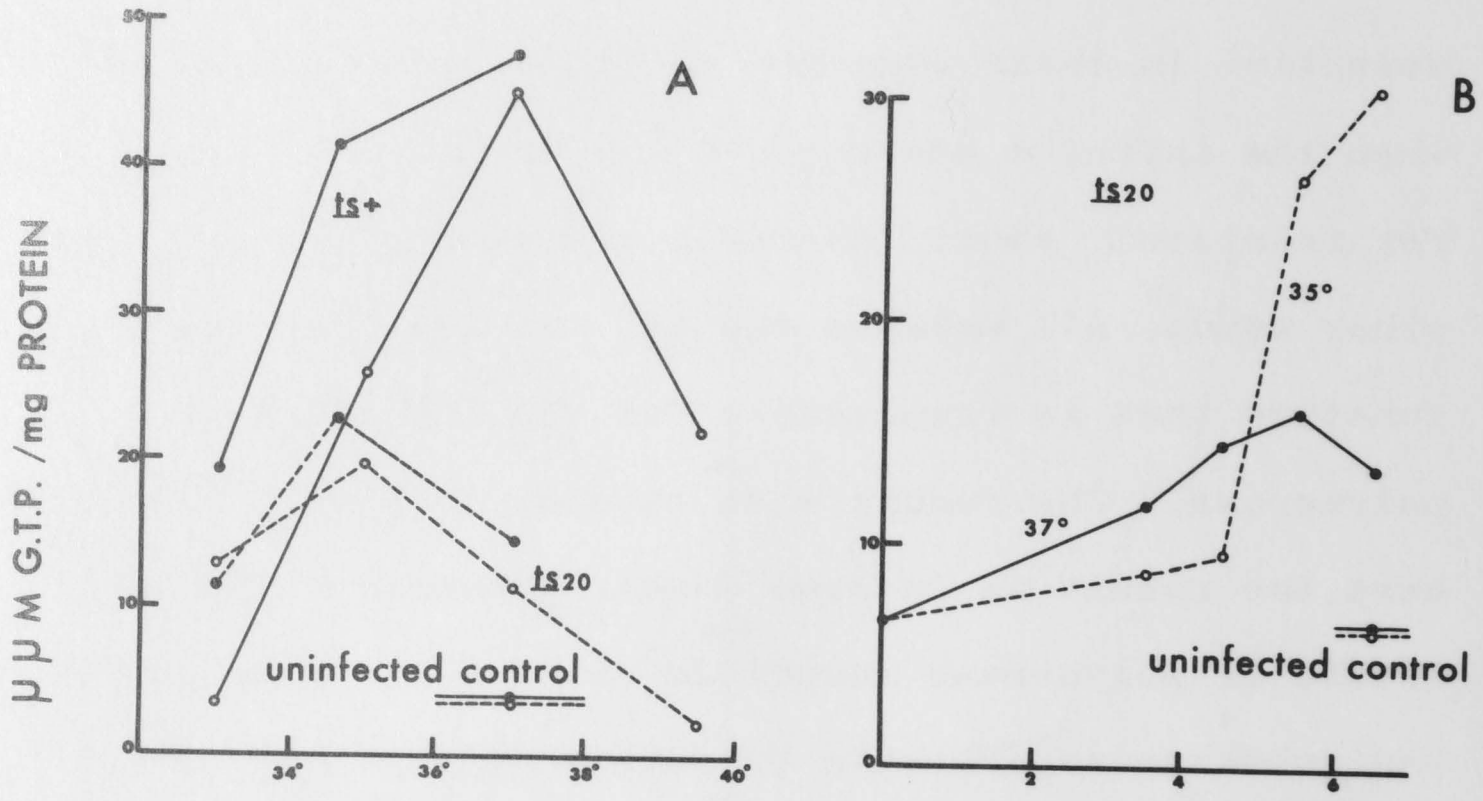


Figure 14: Optimum temperature for polymerase induction in cells infected with ts$^+$ or ts-20. Suspensions of U cells infected with either virus were grown in suspension for 6 hours over a range of temperatures. The cultures were then chilled and polymerase extracts prepared and assayed in the usual manner. The results for two experiments are shown in (A) - denoted by open and closed symbols - and also included are results for uninfected control extracts incubated at 37° C for 6 hours. In (B) the time course of polymerase induction by ts-20 at 35° and 37° C is shown, together with the activity of 6 hour uninfected control preparations for either temperature.

cultures by ts-20 (Table 8), attempts were made to determine the optimum temperature of polymerase induction for ts-20 in comparison with ts^+. Suspensions of 4.0×10^8 U cells were infected with ts-20 and ts^+ at multiplicities of 3-5 p.f.u. per cell and, after adsorption at 4° C, 1.0×10^8 cells from each suspension were added to 100 ml of Spinner Medium in spinner flasks, which were pre-warmed in waterbaths at different temperatures. Incubation at each temperature was continued to 6 hours, when the suspensions were chilled and polymerase preparations of each were made in the usual manner. An uninfected control preparation was made from a 6 hour suspension which was incubated at 37° C, and the activity for all preparations was determined according to standard procedures.

The results of two experiments are shown in Figure 14A. Clearly, much higher levels of polymerase were induced by ts^+ than by ts-20 at all temperatures, and, from the figure, the optimum temperature for induction by ts-20 was $34.5\text{-}35^\circ$ C in comparison with 37° C for ts^+. At the restrictive temperature used in mutant virus assays (39.5° C), the activity of ts-20 polymerase was indistinguishable from that of the uninfected preparation, whereas the activity for ts^+ polymerase was about half of its 37° C value. These findings are consistent with the data in Table 8.

In a similar experiment (data not presented), the optimum temperature for induction of polymerase by ts-3, a protein coat mutant (Table 2), was seen to be the same as for ts^+, although the actual level of polymerase induced by this mutant was lower. A lower optimum temperature of polymerase induction compared with ts^+ may be a feature of RNA negative mutants of poliovirus.

The time course for the synthesis of ts-20 polymerase at 35^o and 37^o C was studied in the manner described for ts^+ at 37.2^o C (Experimental Section 2.1(e)). Polymerase extracts were prepared from samples taken at various times after infection and their activity is shown in Figure 14B.

In this figure the results from the previous experiment are largely confirmed. Considerably higher levels of polymerase are induced at 35^o C than at 37^o C, although at 35^o C maximum levels appeared slightly later. The experiment was concluded at 6.5 hours, and it is possible that levels at 35^o C may have continued to increase past this time. On the basis of both experiments it seems that a thermosensitive event occurs during the induction of polymerase by ts-20, which results in lowered yields of polymerase compared with those induced by ts^+ under identical conditions.

Further evidence of differences between the optimum temperature of polymerase induction for *ts*$^+$ and *ts*-20 was obtained from an experiment in which 5 hour levels of polymerase induced by *ts*$^+$, *ts*-20 and $^+$*R*-20, a revertant of *ts*-20, were compared. Mean activities for replicate samples of *ts*$^+$, *ts*-20 and $^+$*R*-20 polymerase of 75.1, 28.9 and 82.8 μμmoles GTP per mg protein were obtained. The considerable difference in levels of polymerase induced by *ts*$^+$ and $^+$*R*-20, compared with *ts*-20, suggests that a genetic defect in *ts*-20 is responsible for differences in the optimum temperature of polymerase induction noted in Figure 14A.

(d) The thermal stability of polymerases induced by RNA negative mutants of poliovirus

The thermosensitive event in polymerase induction by *ts*-20 (see above) may arise from a mutation in the polymerase gene, or in another gene necessary for enzyme synthesis. Such a mutation could change the secondary structure of the gene products and make them less stable at high temperatures than the corresponding product of *ts*$^+$.

In an examination of these possibilities, the *in vitro* thermal stability of polymerases induced by *ts*$^+$, -20 and -28 were studied in two experiments, which are described in the following sub-sections:

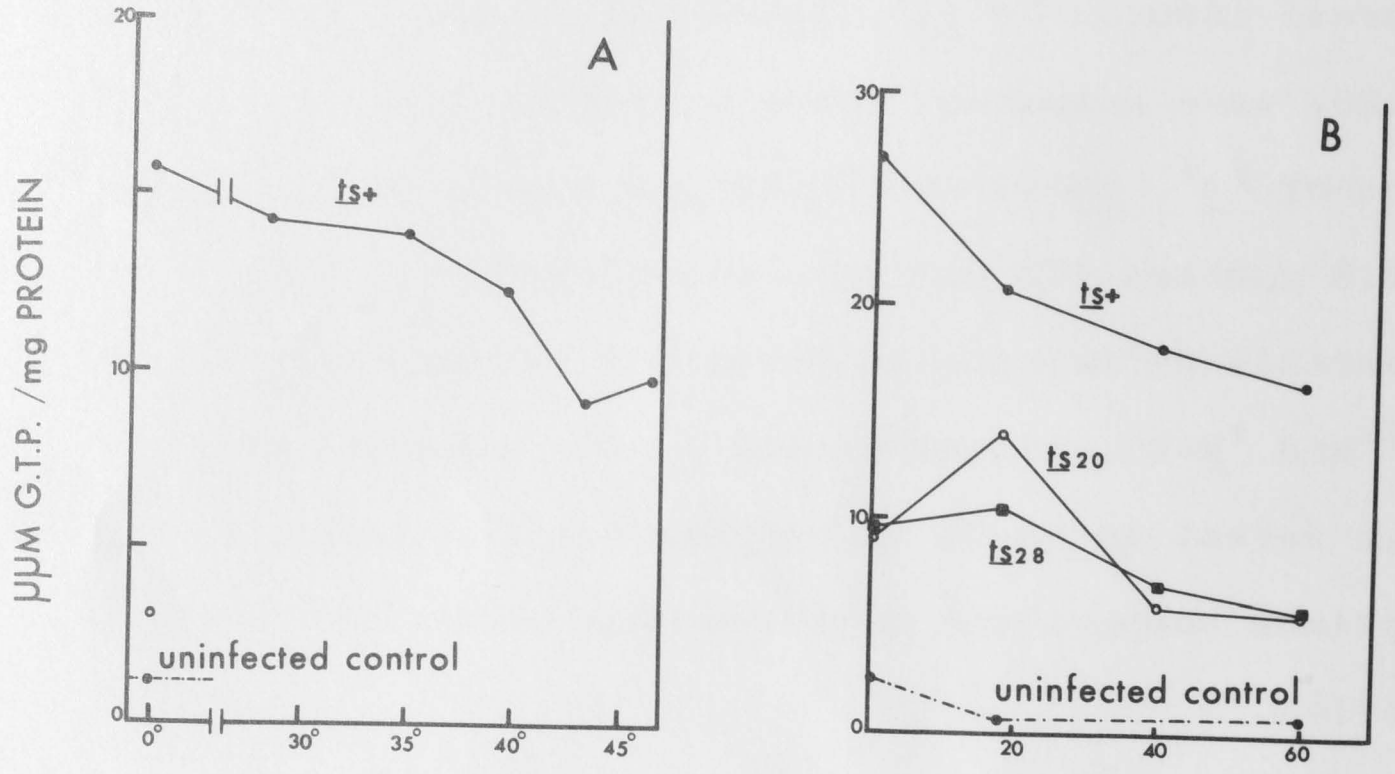


Figure 15: The in vitro thermal stability of polymerases induced by ts^+, ts-20 and ts-28. a 6 hour preparation of ts^+ polymerase, induced at 37.2° C, was heated at various temperatures for 30 minutes and the remaining activity determined (A). Also included in (A) is the level of activity for ts^+ polymerase after induction at 39.5° C (open symbol) and for a 6 hour uninfected control extract prepared at 37.2° C. In (B) 6 hour preparations of polymerases induced by ts^+, ts-20 and ts-28 at 37.2° C and an uninfected control extract were heated to 43° C for varying times and the remaining activity determined in the usual manner.

(i) The effects of preheating polymerase preparations before using in reaction mixtures

As a preliminary to this experiment, the stability of a 6 hour preparation of ts$^+$ polymerase was examined. Replicate samples of the polymerase were treated at various temperatures for 30 minutes and were then chilled. The residual polymerase activity of 0.2 ml samples was then determined by standard procedures and the results of the experiment are presented in Figure 15A.

The results suggest that ts$^+$ polymerase is relatively stable with about a 40 per cent drop in activity occurring only at 43-47^o C. The residual activity at these temperatures was considerably higher than the activity of a 6 hour preparation prepared from the same batch of cells at 39.5^o C, and from an uninfected control extract. On the basis of the experiment a temperature of 43^o C was used in comparative studies of thermostability for other polymerases.

In the second part of this experiment, 6 hour polymerase preparations of ts$^+$, -20 and -28, prepared at 37.2^o C, were heated at 43^o C for varying periods of up to 1 hour. After heating, the samples were chilled and 0.2 ml amounts were tested for surviving activity according to standard procedures. An uninfected control preparation was also

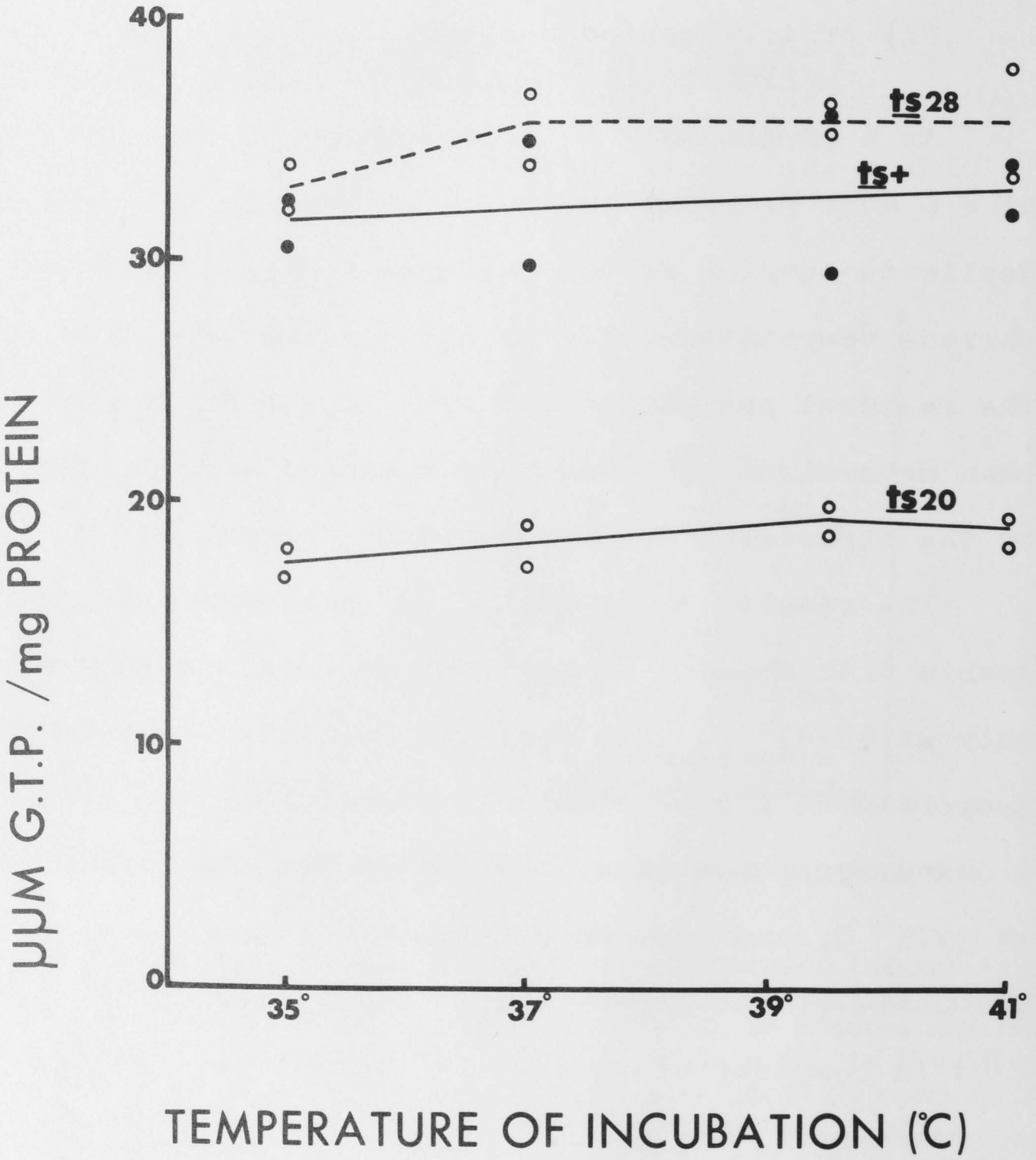


Figure 16: The effects of incubation temperature upon the *in vitro* activity of polymerases induced by ts^+, *ts*-20 and *ts*-28. Replicate reaction mixtures containing 6 hour preparation of ts^+, *ts*-20 and *ts*-28 polymerase (induced at 37.2°C) were incubated at several different temperatures for 30 minutes. The resultant activity determined according to standard procedures.

heated over this period and the results of the experiment are presented in Figure 15B.

From the figure there appear to be no differences between the rates of inactivation for any of the polymerase preparations. This suggests that the thermosensitive step in the synthesis of ts-20 polymerase, noted in the previous section, occurs at a step prior to the synthesis of enzyme, and is not due to lability of the completed molecule.

(ii) The activity of polymerases induced by RNA negative mutants after incubation at different temperatures

A further attempt was made to demonstrate differences in *in vitro* lability between polymerases induced by ts^+, -20 and -28. In this experiment a replicate series of standard reaction mixtures (see Materials and Methods) containing 6 hour polymerase preparations of ts^+, -20 or -28 were incubated for 30 minutes over a range of temperatures. The activity of each mixture was then determined and the results of the experiment are presented in Figure 16.

No loss in activity for the polymerase of ts^+ or either mutant could be detected at any temperature of incubation between 35 and 41° C. In a separate experiment (data not presented) the kinetics of incorporation during

the first 15 minutes of incubation at temperatures between 29° and 41° C, was studied for the polymerases of ts$^+$ and ts-20, but here again no differences were noted. These findings confirm those obtained from Figure 15, namely that, once synthesised, the polymerases of ts$^+$, -20 and -28 are uniformly stable.

Summary

A polymerase assay for poliovirus is described and, in the first part of the section, the characteristics of the enzyme (or enzymes) induced by strain ts$^+$ are outlined. Maximum levels of polymerase were obtained after 6 hours of incubation at 37.2° C and the RNA produced consisted of a complex of single- and double- stranded material. The double stranded RNA of the complex had similar thermal transition properties and a sedimentation coefficient to *in vivo* labelled Replicative Form RNA.

All ts mutant polymerases and that of ts$^+$ produced lower levels of RNA when prepared from cells after growth at 39.5° C for relatively short periods. The optimum temperature for the induction of ts-20 polymerase was shown to be 35° C, in comparison with 37° C for that of ts$^+$. *In vitro* studies indicated that the thermosensitivity occurred at a precursory stage in the synthesis of the enzyme molecule.

EXPERIMENTAL SECTION 3

The Estimation of the Molecular Weight of Poliovirus RNA

In this section further estimates of the molecular weight of poliovirus RNA were obtained by comparing poliovirus RNA with a number of well characterised RNA markers. The following methods of comparison were used: 1 - centrifugation through sucrose gradients under conditions in which RNA secondary structure was minimised, and 2 - electrophoresis in polyacrylamide gels. The preparation of the various radioactive markers has been described in Materials and Methods.

3.1 Centrifugation Studies

(a) Comparisons between the RNA of poliovirus and tobacco mosaic virus (TMV)

It is commonly assumed that the RNA of poliovirus has a molecular weight of 2.0×10^6 daltons (Cooper, 1969). Therefore, poliovirus RNA was compared with TMV RNA, which is probably the best characterised of all RNA molecules and consists of a single length of RNA of molecular weight 2.0×10^6 daltons, when determined by the accurate light scattering technique (Boedtker, 1960).

(i) The effects of formaldehyde upon the sedimentation properties of poliovirus and TMV RNA

Preparations of purified [^{3}H] adenosine TMV and [^{14}C] uridine poliovirus were mixed and the RNA extracted by the single-phase phenol method referred to in Materials and Methods. Sixty micrograms of yeast carrier RNA and one tenth volume of 2M sodium chloride were then added and the total RNA was precipitated with ethanol and allowed to aggregate for 2 hours in an ice bath. The RNA precipitate was collected by centrifugation and dissolved at 4° C in 1 ml of buffer consisting of 0.01 M EDTA and 0.01 M Tris, pH 7.4. Neutral formaldehyde was added to give a final concentration of 6 per cent (W/V) to two 0.2 ml amounts of the RNA preparation, and the mixture heated in a thermostatically controlled waterbath at 60° C for 1 hour. Under these conditions most RNA secondary structures are eliminated (Haselkorn and Doty, 1961; Fenwick, 1968). The two formaldehyde-treated preparations were then centrifuged through 15-30 per cent (W/V) sucrose gradients in the presence and absence of 6 per cent formaldehyde and 0.1 M sodium chloride (C and D) as indicated in the legend for Figure 17. The influence of 0.1 M sodium chloride as a determinant of RNA secondary structure was also studied (A and B). Gradients C and D were centrifuged at the one time.

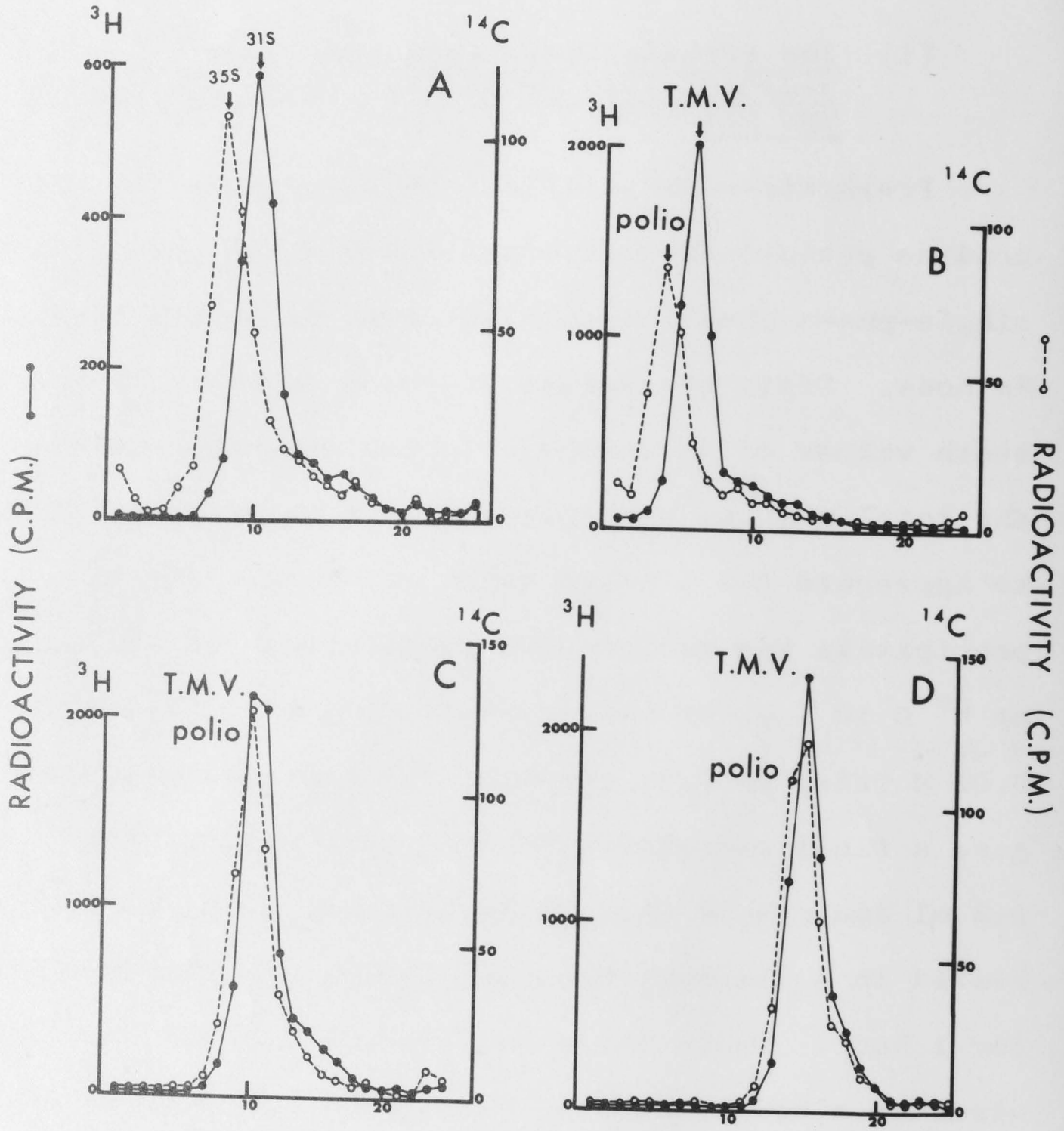


Figure 17: Sucrose-gradient analysis of [^{14}C] poliovirus and [^{3}H] TMV RNA in the presence and absence of 6 per cent formaldehyde and 0.1 M sodium chloride. Four fractions (A, B, C and D) of a mixture of poliovirus and TMV RNA, prepared from purified virions, were treated as follows:

Sodium chloride to 0.1 M was added to (A), which was then centrifuged through 15-30 per cent sucrose gradients containing 0.1 M sodium chloride for 4 hours at 39,500 r.p.m.. Fraction (B) was centrifuged for 7 hours in the same manner in the absence of sodium chloride. Fraction (C) was heated for 1 hour at 60° C with 6 per cent formaldehyde. Sodium chloride to 0.1 M was then added and the mixture centrifuged for 7 hours through sucrose gradients containing 6 per cent formaldehyde and 0.1 M sodium chloride. Fraction (D) was treated as for (C), except that sodium chloride was omitted from the mixture and the gradient. The gradient distribution of acid-insoluble [^{14}C] poliovirus RNA (broken lines) and [^{3}H] TMV RNA (solid lines) is shown.

After centrifugation, 0.2 ml fractions of the gradient were collected onto paper strips, which were acid washed as described in Materials and Methods and the radioactivity present on each determined.

The results of this experiment (Figure 17) indicate that, in the absence of formaldehyde, poliovirus RNA sediments more rapidly in sucrose gradients than TMV RNA. A similar relationship was obtained when the two RNA species were centrifuged in the absence of sodium chloride, suggesting that RNA secondary structures were still present. The relative position of both types of RNA in gradients A and B accords with observed values of 35 and 30.8S for the sedimentation coefficients of type I poliovirus and TMV RNA, respectively, in the presence of 0.1 M sodium chloride (Baltimore and Girard, 1966; Fenwick, 1968).

In the presence of formaldehyde (gradients C and D), identical peaks for both RNA species were obtained irrespective of whether sodium chloride was present or not. However in both instances, the leading edge of the curve for poliovirus RNA appeared slightly ahead of that for TMV, suggesting that slight differences in sedimentation coefficient still exist. The sedimentation coefficients of both poliovirus and TMV RNA, after formaldehyde

treatment, were smaller in the absence than in the presence of 0.1 M sodium chloride. Fenwick (1968) suggests that sodium ions permit folding of the formylated chain by shielding the negative charges of phosphate groups and, under these conditions, has observed a linear relationship between sedimentation coefficient and molecular weight for a number of RNA species examined, except mammalian '28S' ribosomal RNA. Boedtker (1968 a) has observed a similar relationship for several RNA species but these did not include '28S' ribosomal RNA.

Because of the coincidence of the peaks in Figure 17 C and D, it is not possible to measure accurately any small difference that may be present between the sedimentation coefficients of poliovirus and TMV RNA after formaldehyde treatment. Slight differences, however, do appear to be present, and relating sedimentation coefficient in the presence of formaldehyde directly with molecular weight (Fenwick, 1968; Boedtker, 1968 a), poliovirus RNA would appear to have a molecular weight slightly in excess of 2.0×10^6 daltons (i.e. about $2.1 - 2.2 \times 10^6$ daltons). However, in Experimental Section 3.1 b, relatively poor resolution was obtained in sucrose-formaldehyde gradients containing no sodium chloride

between the RNA of poliovirus and bacteriophage R17, despite an anticipated twofold difference in their molecular weights. As a consequence, the difference in the molecular weights of poliovirus and TMV RNA may be greater than is apparent from Figure 17 C and D.

(ii) The use of dimethyl sulphoxide (DMSO) in comparative sedimentation analyses of poliovirus and TMV RNA

Attempts were made to confirm the previous findings for the RNA of poliovirus and TMV by centrifugation through sucrose-DMSO gradients. DMSO is a powerful denaturant of RNA which may be used either to disrupt internal secondary structure, or to bring about the separation of the strands of RNA duplexes (Katz and Penman, 1966; Strauss et al., 1968). The RNA can be treated with DMSO at room temperature, thereby greatly decreasing the possibility of thermal scissions within RNA molecules that could occur during heating at 60^{o} C in the presence of formaldehyde.

Mixtures of [^{3}H] adenosine TMV and [^{14}C] uridine poliovirus RNA were prepared and were precipitated with ethanol as described in the previous section, and the RNA precipitate dissolved in 0.2 ml of buffer consisting of 0.01 M EDTA, 0.01 M Tris, pH 7.4. Fifty microlitres of the mixed preparation were added to a fivefold excess of

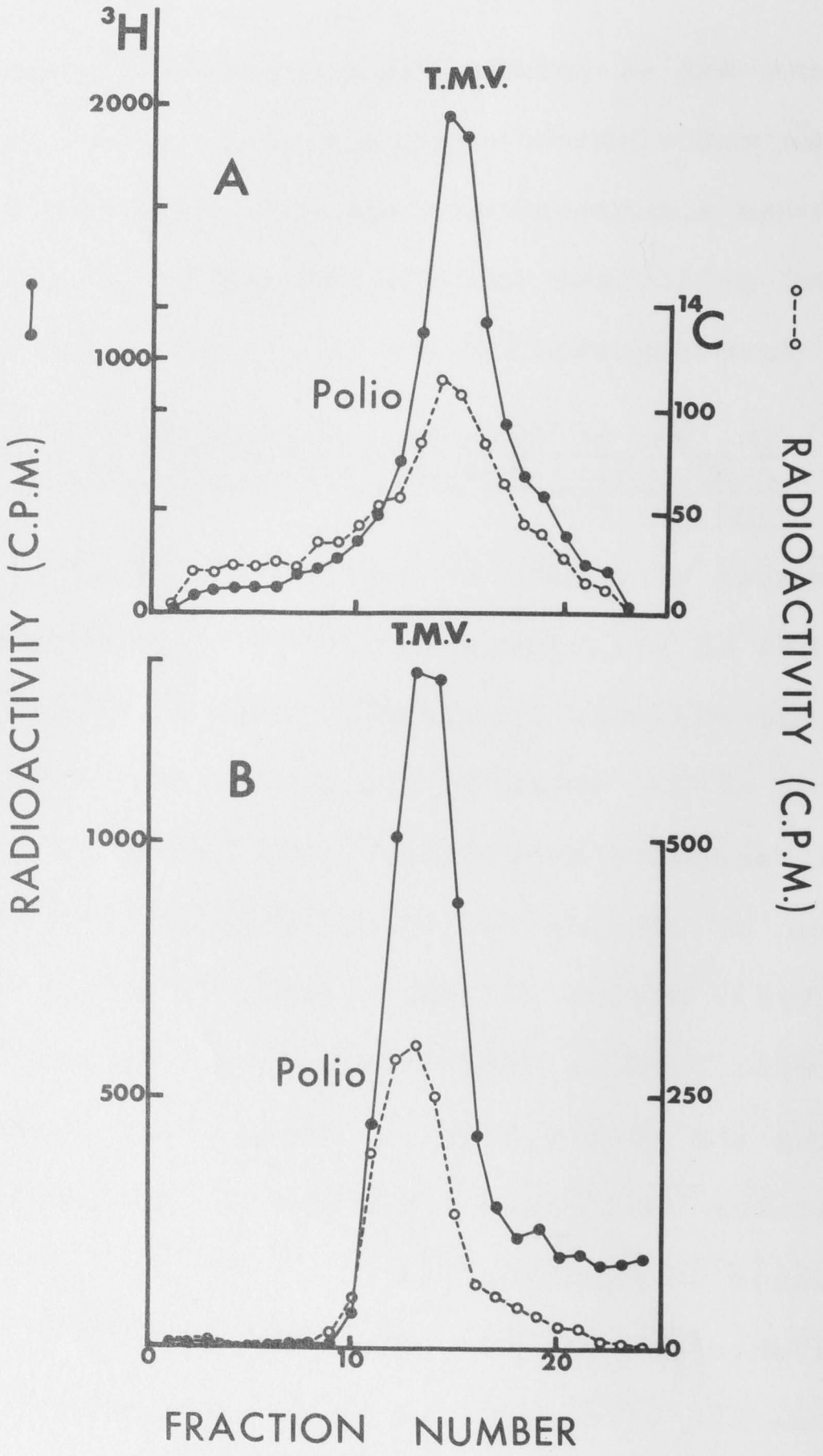


Figure 18: Sucrose-DMSO gradient analysis of mixtures of [^{14}C] poliovirus and [^{3}H] TMV RNA. Two RNA mixtures (A and B) were obtained from purified virions, as described in the text. Mixture A was treated with a fivefold excess of DMSO for 30 minutes at room temperature and centrifuged through 5-20 per cent sucrose gradients prepared in 99 per cent DMSO and containing 10 mM lithium chloride for 5 hours at 65,000 r.p.m.. Mixture B was treated with 6 per cent formaldehyde at 60° C for 1 hour, treated with DMSO and centrifuged for 7 hours at 65,000 r.p.m. through similar gradients. The gradient distribution of [^{14}C] poliovirus (broken lines) and [^{3}H] TMV RNA (solid lines) is shown.

99 per cent DMSO to produce mixture A, which was then allowed to stand at room temperature for 30 minutes. A second sample of 50 µl was treated, in addition, with 6 per cent formaldehyde and heated at 60° C for 1 hour. A fivefold excess of DMSO was then added to produce mixture B, which was also allowed to stand for 30 minutes.

Both RNA preparations were then centrifuged at 23° C through pre-formed 4 ml sucrose gradients (5-20 per cent W/V), prepared in 99 per cent DMSO containing 10 mM lithium chloride, using a Spinco SW-65 rotor. Mixture A was centrifuged for 5 hours and mixture B for 7 hours at 65,000 r.p.m.. Fractions of 0.2 ml were collected into scintillation vials and 10 ml of the scintillation fluid used in gel electrophoresis studies (see Materials and Methods) was added to each, and the radioactivity present determined.

The sedimentation profiles for RNA mixtures A and B in DMSO are presented in Figure 18. Coincident peaks of poliovirus and TMV RNA were obtained from both gradients, but, where the RNA preparations were treated in addition with formaldehyde, the distribution of radioactivity for poliovirus RNA appeared slightly ahead of that for TMV RNA. A more coincident distribution of radioactivity for both RNA species appeared after treatment with DMSO alone.

Strauss et al. (1968) have demonstrated a linear logarithmic relationship between sedimentation coefficient and molecular weight in the presence of 99 per cent DMSO. The results in gradient A suggest that both poliovirus and TMV RNA have identical molecular weights, but gradient B tends to confirm the previous estimate for poliovirus RNA as being slightly in excess of that for TMV RNA.

It was necessary to centrifuge the RNA mixture after treatment with both DMSO and formaldehyde for 7 hours compared with 5 hours after DMSO alone, in order for adequate movement of RNA into the gradient to occur. This may reflect a slight superiority of formaldehyde over DMSO as an RNA denaturant, under the conditions of use in the present and previous experiments.

(b) Comparisons between the RNA of poliovirus and bacteriophage R17

The sedimentation properties of the RNA of poliovirus and bacteriophage R17 were compared after treatment with formaldehyde. R17 RNA has a widely accepted value of 1.1×10^6 daltons when determined by light scattering (Gesteland and Boedtker, 1964). RNA extracts were prepared from mixtures of $[^{14}C]$ uridine poliovirus and $[^{3}H]$ adenosine R17, as described in section 3.1 a(i), and the mixture was heated in the presence of 6 per cent

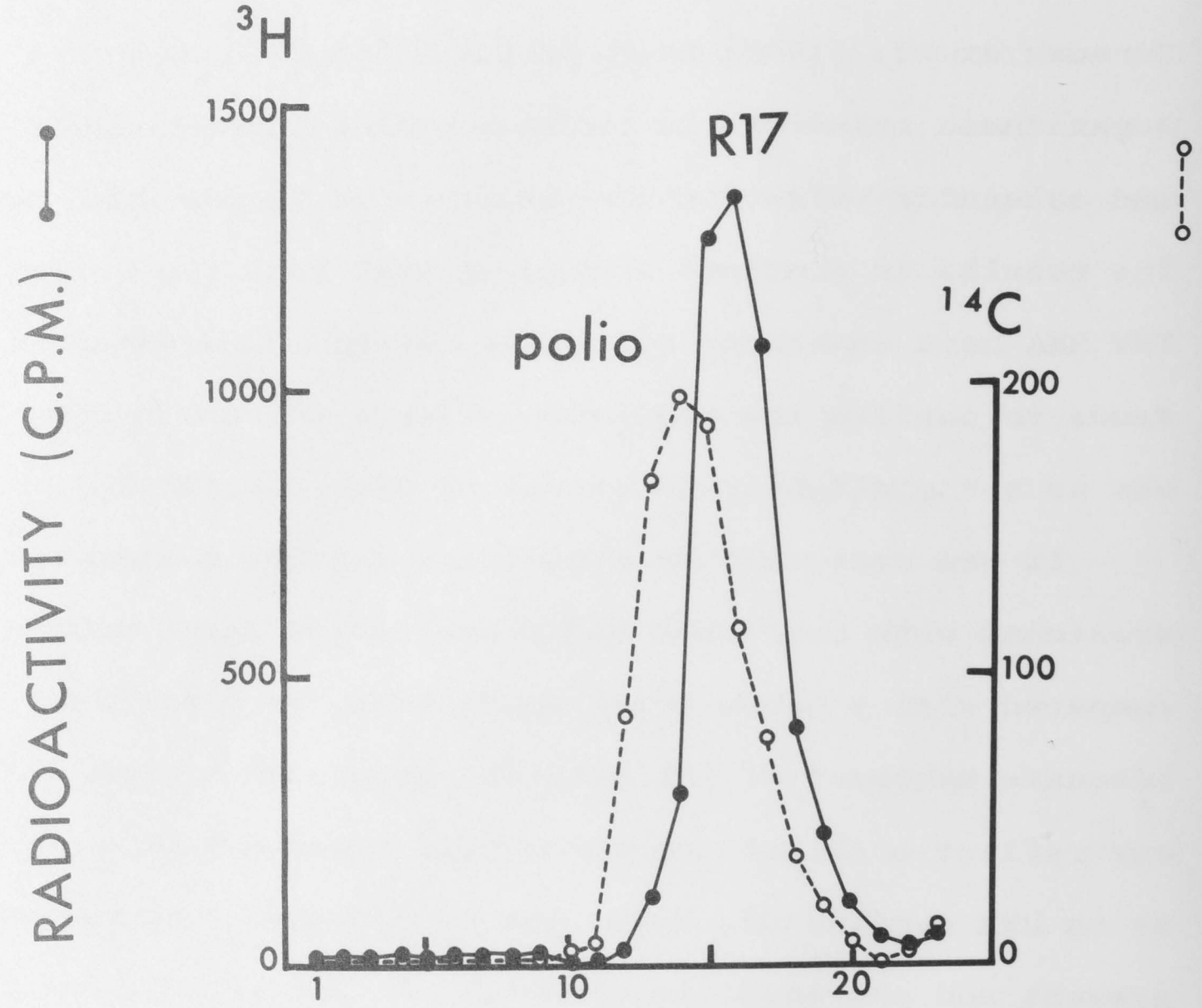


Figure 19: Sucrose-formaldehyde gradient analysis of a mixture of [^{14}C] poliovirus and [^{3}H] R17 RNA. A mixture of both types of RNA was obtained as described in the text and was heated to 60° C for 1 hour with 6 per cent formaldehyde. The mixture was then centrifuged through 15-30 per cent sucrose gradients containing 6 per cent formaldehyde but no sodium chloride. The gradient distribution of acid-insoluble [^{14}C] poliovirus (broken line) and [^{3}H] R17 RNA (solid line) is presented.

formaldehyde for 1 hour at 60° C. The preparation was then centrifuged for 7 hours at 39,500 r.p.m. through 15-30 per cent sucrose-formaldehyde gradients containing no sodium chloride, using an SW-39 rotor at 7° C. Fractions were collected in the usual manner onto paper strips which were acid washed and the radioactivity present on each determined.

The results in Figure 19 show clearly that poliovirus RNA has a higher sedimentation coefficient than that of R17 after treatment with formaldehyde. However, the separation of the two RNA types in the gradient was less than expected, in view of an anticipated twofold difference in their molecular weights. Thus the resolving power of 15-30 per cent sucrose-formaldehyde gradients, containing no sodium chloride, may not be sufficiently great to detect small differences in molecular weight, such as probably exist between the RNA of TMV and poliovirus (see previous sections). Greater resolution could possibly be achieved by the use of steeper gradients.

(c) Comparative Studies with the RNA of poliovirus and Semliki Forest virus (SFV)

In the following experiments, attempts were made to compare the molecular weights of poliovirus and SFV RNA under conditions where RNA secondary structure was

minimised. SFV is a group A arbovirus whose RNA has a reported sedimentation coefficient of 40-45S (Friedman, Levy and Carter, 1966; Sonnabend, Martin and Mecs, 1967). By applying the empirical relationship of Spirin (1961), SFV RNA would have a molecular weight in excess of 3×10^6 daltons. However, suggestions have been made (Sonnabend, Mecs and Martin, 1966) that 40-45S RNA is a more compact form of interjacent 26S RNA, which is present in variable amounts in SFV infected cells.

Good evidence that such is the case for the RNA of Western equine encephalitis virus (WEE), another group A arbovirus, was presented by Sreevalsan, Lockart, Dodson and Hartman (1968). They were able to convert the 40S form of WEE RNA into 26S interjacent RNA by (a) heating to 90° C and cooling rapidly (b) dialysing against distilled water or (c) treatment with 8M urea or DMSO. They showed that both 26S and 40S RNA had identical base compositions and densities, and were able to convert about 50 per cent of 26S RNA into 40S RNA by dialysis against 0.5 M sodium chloride or 0.05 M acetate buffer, pH 4.0.

Preparations of [^{3}H] adenosine SFV RNA were obtained by incubating suspensions of purified labelled virions and SDS (final concentration 1 per cent) for 30 minutes at 37° C (extraction yields for SFV using the single-phase

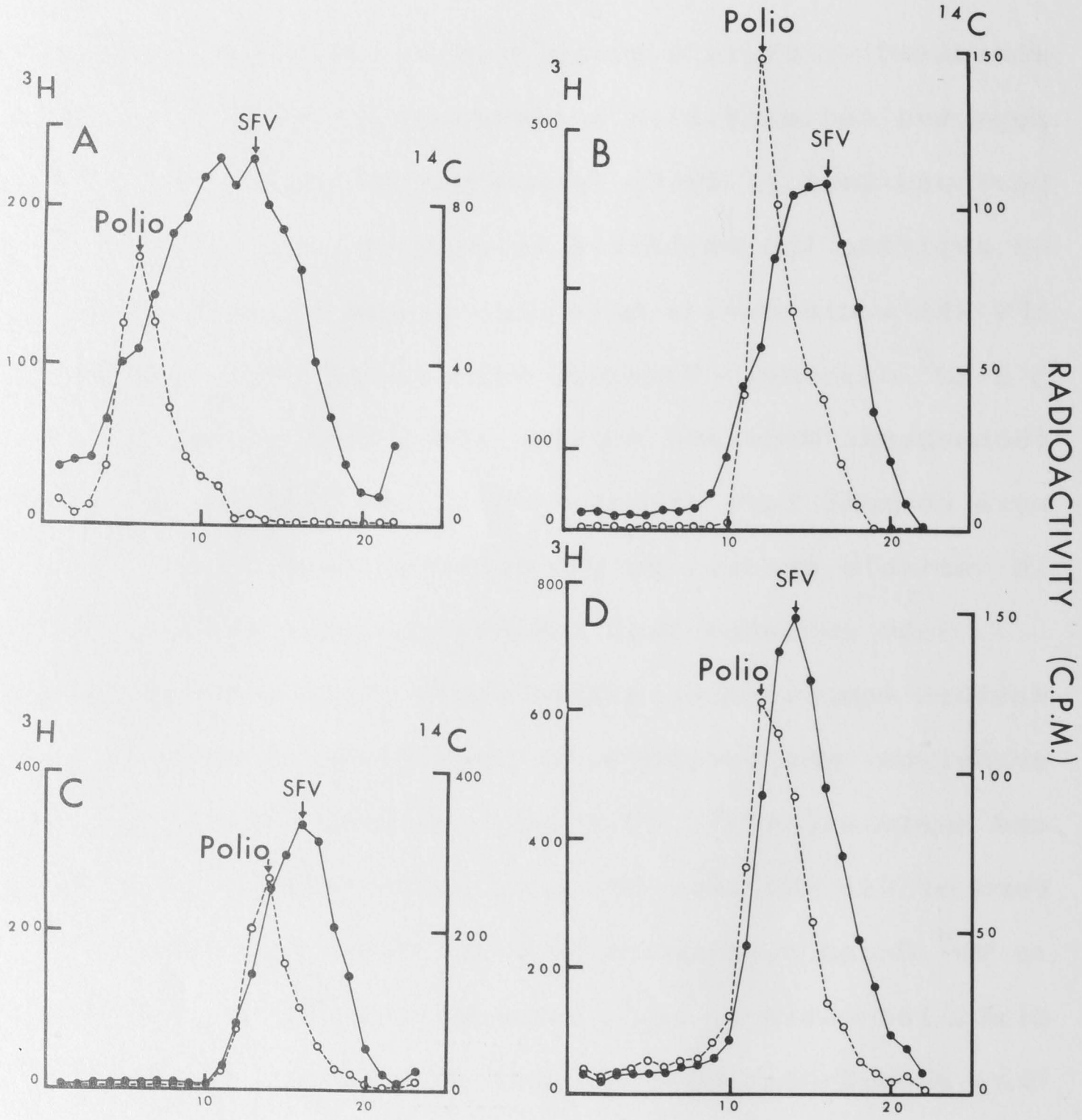


Figure 20: Sucrose-gradient analysis of [^{14}C] poliovirus and [^{3}H] SFV RNA in the presence and absence of formaldehyde or DMSO. A mixture of both RNA species was obtained as described in the text and was divided into 4 fractions (A, B, C and D). The procedures used were those outlined for treatment with and centrifugation in the presence of formaldehyde or DMSO in the legends for Figures 17-19. Fraction A was centrifuged through sucrose gradients containing 0.1 M sodium chloride for 4 hours at 39,500 r.p.m. Fraction B was centrifuged through similar gradients containing 6 per cent formaldehyde for 7 hours after formaldehyde treatment. Fraction C was treated and centrifuged as for B except that sodium chloride was omitted, while Fraction D was centrifuged through a 5-20 per cent DMSO gradient for 5 hours at 65,000 r.p.m. The gradient distributions of [^{14}C] poliovirus (broken lines) and [^{3}H] SFV (solid lines) are presented.

phenol method were relatively poor). The RNA was then mixed with a suspension of purified [^{14}C] uridine poliovirions and the total mixture extracted by the single-phase phenol method. Carrier yeast RNA was added and the total RNA precipitated with ethanol as described in 3.1 a(i), and finally dissolved in a small volume of buffer consisting of 0.01 M EDTA and 0.01 M Tris, pH 7.4.

Two 100 µl amounts of this mixture were then treated with formaldehyde at 60° C, and centrifuged through sucrose-formaldehyde gradients in the presence or absence of 0.1 M sodium chloride (B and C). Another 100 µl amount was treated with DMSO, as described in 3.1 a(ii), and centrifuged through sucrose-DMSO gradients (D), while, as a control, a fourth untreated preparation was centrifuged through gradients containing 0.1 M sodium chloride (A).

The results are presented in Figure 20. Gradient A suggests that SFV RNA, after extraction, was heterogeneous in its sedimentation behaviour, with a position relative to poliovirus RNA similar to that of interjacent RNA (Sonnabend et al., 1966). This heterogeneity was partly eliminated after centrifugation through sucrose-formaldehyde gradients (B and C) but good resolution was

achieved with the DMSO gradient (D). With DMSO, a sedimentation coefficient considerably lower than poliovirus RNA can be observed, and, relating this to molecular weight, a value of between 1 and 2 x 10^6 daltons would appear to be the molecular weight of SFV RNA. This figure is about half the value anticipated by applying the 40-45S sedimentation coefficient to Spirin's formula.

In several experiments, only the 26S form of SFV RNA, shown in Figure 20, was obtained after extraction. The inability to obtain 40-45S RNA may be due to unknown features of the particular extraction procedure, or to ethanol precipitation, in bringing about the disruption of secondary structures normally present in this form of RNA.

(d) Comparative Studies using Formaldehyde and DMSO

Fenwick (1968) found that preparations of the larger ribosomal ('28S') RNA component of HeLa cells, after treatment with formaldehyde, had a sedimentation coefficient greater than TMV RNA. This finding was unexpected, in view of a reported molecular weight of 1.65 x 10^6 daltons for '28S' RNA, obtained by equilibrium centrifugation (Hamilton, 1967) compared with 2.0 x 10^6 for TMV RNA, and may be due to non-linear structures present in '28S' RNA either before or after formaldehyde

treatment (Fenwick, 1968). However no such anomalies were noted by Strauss et al. (1969) after treatment of rabbit reticulocyte ribosomal RNA and TMV RNA with 99 per cent DMSO. Therefore the following experiment was done to compare the effects of both RNA denaturants in parallel, and to confirm Fenwick's findings.

A preparation of [^{3}H] adenosine TMV RNA, obtained by the single-phase phenol method, was mixed with 0.2 ml of [^{14}C] uridine U-cell ribosomal RNA. Yeast carrier RNA was added and the total RNA precipitated with ethanol and redissolved in 0.4 ml of buffer consisting of 0.01 M EDTA, 0.01 M Tris, pH 7.4.

Sodium chloride to 0.1 M was added to 100 µl of the preparation (mixture A), while a similar sample was heated in the presence of 6 per cent formaldehyde at 60^{o} C for 1 hour (mixture B). A fivefold excess of DMSO was added to a third sample (C) and the mixture allowed to stand at room temperature for 30 minutes. Mixtures A, B and C were then centrifuged through sucrose gradients prepared in 0.1 M sodium chloride, 6 per cent formaldehyde and 99 per cent DMSO, respectively, for 4, 7 and 5 hours, under conditions outlined in the previous sections. Fractions were collected and the radioactivity present determined.

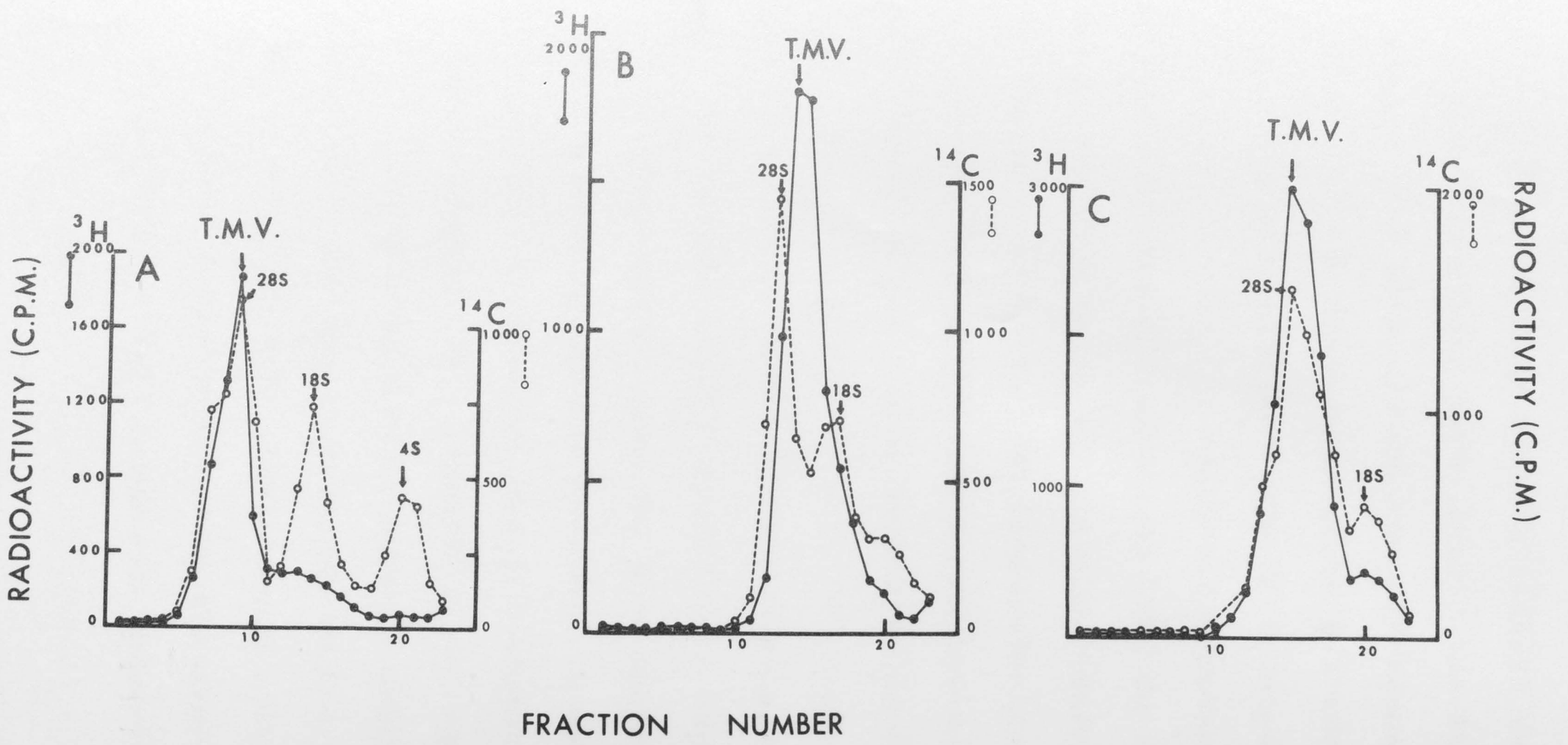


Figure 21: Comparative sucrose-formaldehyde and sucrose-DMSO gradient analyses of a mixture of [^{14}C] U cell ribosomal and [^{3}H] TMV RNA. A mixture of both RNAs was prepared as described in the text and was divided into 3 fractions (A, B and C). Sodium chloride to 0.1 M was added to fraction (A) which was then centrifuged for 4 hours through 15-30 per cent sucrose gradients containing 0.1 M sodium chloride. Fraction (B) was heated with 6 per cent formaldehyde for 1 hour at 60^{o} C and centrifuged for 7 hours through 15-30 per cent sucrose gradients containing 6 per cent formaldehyde. Fraction (C) was treated with a fivefold excess of DMSO and centrifuged for 5 hours through a 5-20 per cent sucrose gradient, prepared in DMSO and containing 10 m M lithium chloride. The gradient distribution of [^{14}C] ribosomal RNA (broken lines) and [^{3}H] TMV (solid lines) is presented.

The results in Figure 21 suggest that in the presence of 0.1 M sodium chloride (A) or DMSO (C) the larger ribosomal RNA component has a sedimentation coefficient identical with TMV RNA, which was estimated by Fenwick (1968), in the presence of 0.1 M sodium chloride, to be 30.8 S. After treatment with formaldehyde (C), the sedimentation coefficient for '28S' ribosomal RNA is greater than that for TMV RNA, thus confirming Fenwick's findings.

Both findings for the RNA denaturants are unexpected in view of the reported molecular weights for the two RNA species. This could be due to non-linear secondary structures in the '28S' RNA, as Fenwick suggests, or it may be that the value of 1.65×10^6 daltons assigned to its molecular weight is an underestimate of the true figure. The sharp resolution of the RNA peaks after treatment with formaldehyde in Figure 21 B is not consistent with a high degree of internal secondary structure that might be expected if a branched molecule was present. The peak obtained after formaldehyde treatment is, in fact, better resolved than peaks obtained with SFV RNA (Figure 20), after similar treatment. Untreated SFV RNA has considerable internal secondary structure (Sonnabend et al., 1966).

On the basis of this and a previous experiment where preparations of poliovirus and TMV were treated with both formaldehyde and DMSO (Figure 18 B), heating to 60° C in the presence of formaldehyde would appear to bring about more effective RNA denaturation than treatment with DMSO alone.

3.2 Studies Using Polyacrylamide Gel Electrophoresis

(a) Comparative studies with poliovirus RNA and other RNA species

In this section, the electrophoretic mobility of poliovirus RNA was compared with that of TMV RNA and a number of other RNA markers. RNA was extracted from suspensions of [^{14}C] uridine poliovirus particles and other radioactive virus preparations by the single-phase phenol technique. After extraction, 0.2 ml of a [^{14}C] uridine ribosomal RNA preparation (see Materials and Methods) was added as required. Yeast carrier RNA was then added and the total RNA precipitated with ethanol, redissolved in buffer E (see Materials and Methods) and submitted to electrophoresis for 4 hours, as described in Materials and Methods.

The patterns obtained after electrophoresis of three RNA mixtures are shown in Figure 22. Mixture A consisted of poliovirus and TMV RNA; mixture B contained, in

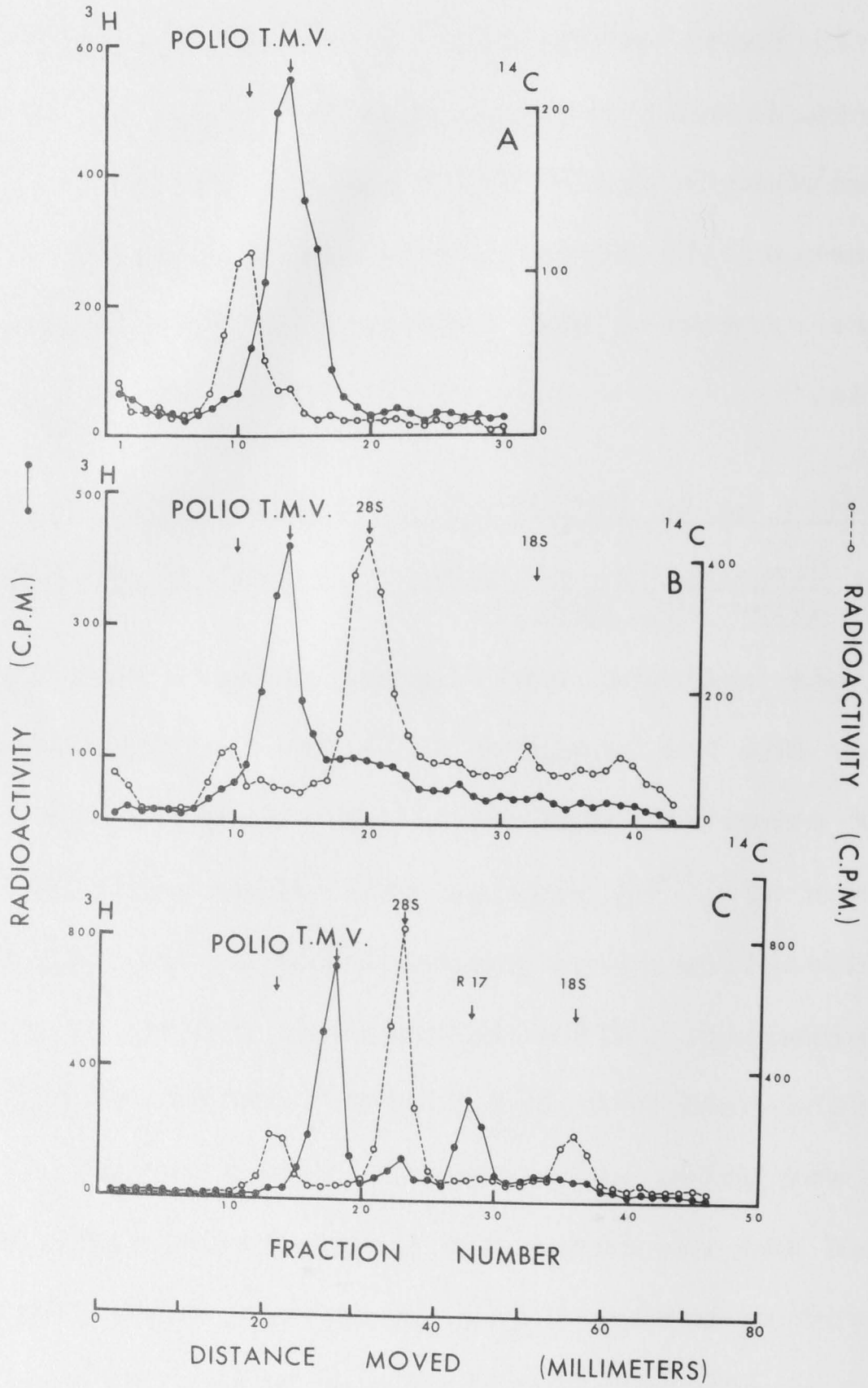


Figure 22: Polyacrylamide gel electrophoresis of mixtures of [^{14}C] poliovirus RNA and other RNA marker preparations. Mixtures of [^{14}C] poliovirus, [^{3}H] TMV, [^{14}C] ribosomal and [^{3}H] R17 RNA were prepared as described in the text and submitted to electrophoresis for 4 hours in 2.4 per cent polyacrylamide gels, at 70 volts and 6 mA per tube. The gels were then sliced and the radioactivity present determined, as described in Materials and Methods.

addition, ribosomal RNA, while mixture C consisted of poliovirus, TMV, ribosomal and R17 RNA. Mixture C was run in duplicate, but the results for only one gel are presented in Figure 22.

The figure shows that poliovirus RNA has a lower electroporetic mobility than that of TMV or any other species examined, and, from the findings of Bishop et al. (1967), relating RNA molecular weight with mobility, this would imply that poliovirus RNA had a molecular weight considerably in excess of 2.0×10^6 daltons.

A linear relationship can be seen in Figure 23, when data obtained for RNA mixture C in replicate experiments was plotted semi-logarithmically against the known molecular weights of the various RNA markers used. The molecular weight for poliovirus RNA, obtained by extrapolation, is 2.65×10^6 daltons. When the data for mixture B is plotted in similar fashion an extrapolated value of 2.4×10^6 was obtained for poliovirus RNA.

There are, unfortunately, very few well characterised RNA markers of molecular weight $2.5 - 3.0 \times 10^6$ daltons that could be used in similar confirmatory studies. However, it is clear from Figure 23, that the various RNA markers used behave in the linear manner described by Bishop et al., 1967. Of particular interest is the

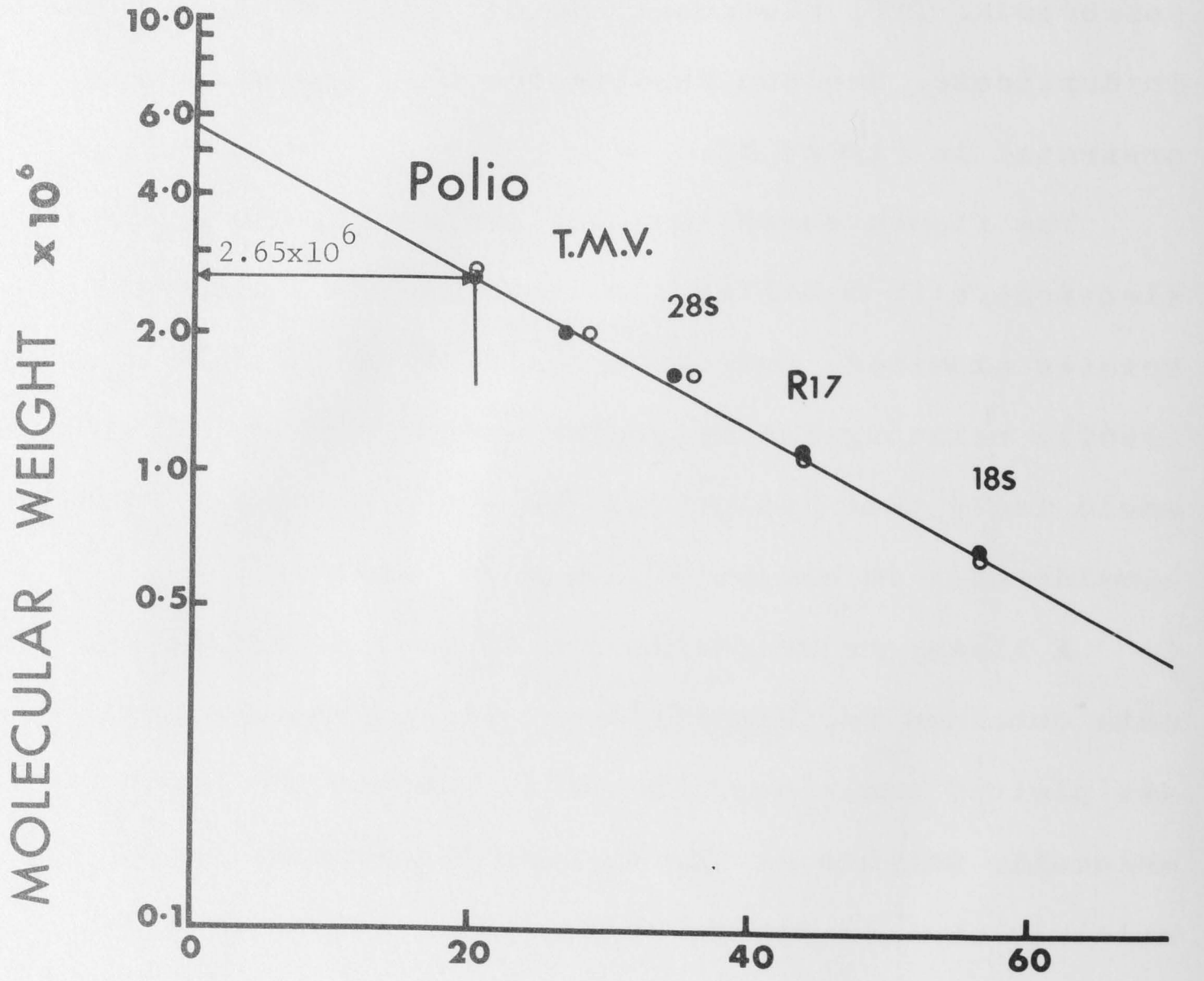


Figure 23: The relationship between RNA molecular weight and electrophoretic mobility in 2.4 per cent polyacrylamide gels. The data from Figure 22C and from a replicate gel run simultaneously were obtained, and the distance moved by the various RNA species is plotted against the logarithm of their known molecular weights. The molecular weights accepted for the various RNA markers are: TMV RNA 2.0 x 10^6 daltons (Boedtker, 1960), '28'S and '18'S ribosomal marker RNA, 1.64 x 10^6 and 0.67 x 10^6 daltons, respectively (Petermann and Pavlovec, 1966) R17 RNA, 1.1 x 10^6 daltons (Gesteland and Boedtker, 1964). The molecular weight for poliovirus RNA, obtained by extrapolation, was 2.65 x 10^6 daltons.

position of R17 RNA relative to that of TMV RNA. Gesteland and Boedtker, (1964) observed that, despite large differences in molecular weight, the sedimentation coefficients of R17 and TMV were very similar - due to extensive base pairing within the R17 RNA molecule. From Figure 23, the electrophoretic mobility of R17 RNA, relative to the other RNA species, appears to be proportional to its molecular weight.

(b) The effects of removal of RNA secondary structure upon electrophoretic mobility

The electrophoretic mobilities of poliovirus and TMV RNA were compared after treatment with formaldehyde. Mixtures of [^{14}C] uridine poliovirus and [^{3}H] adenosine TMV RNA were prepared by the single-phase phenol method and were precipitated with ethanol after the addition of carrier RNA. The RNA was dissolved in a small volume of electrophoresis buffer, and neutral formaldehyde to 6 per cent was then added to part of the sample. The mixture was heated at 60^{o} C for 1 hour; under these conditions most viral RNA secondary structure have been reported to be removed (Fenwick, 1968; Boedtker, 1968 a).

Two 70 µl amounts of formaldehyde-treated RNA and a similar volume of untreated material were than placed on 5 cm 2.4 per cent polyacrylamide gels and electrophoresed

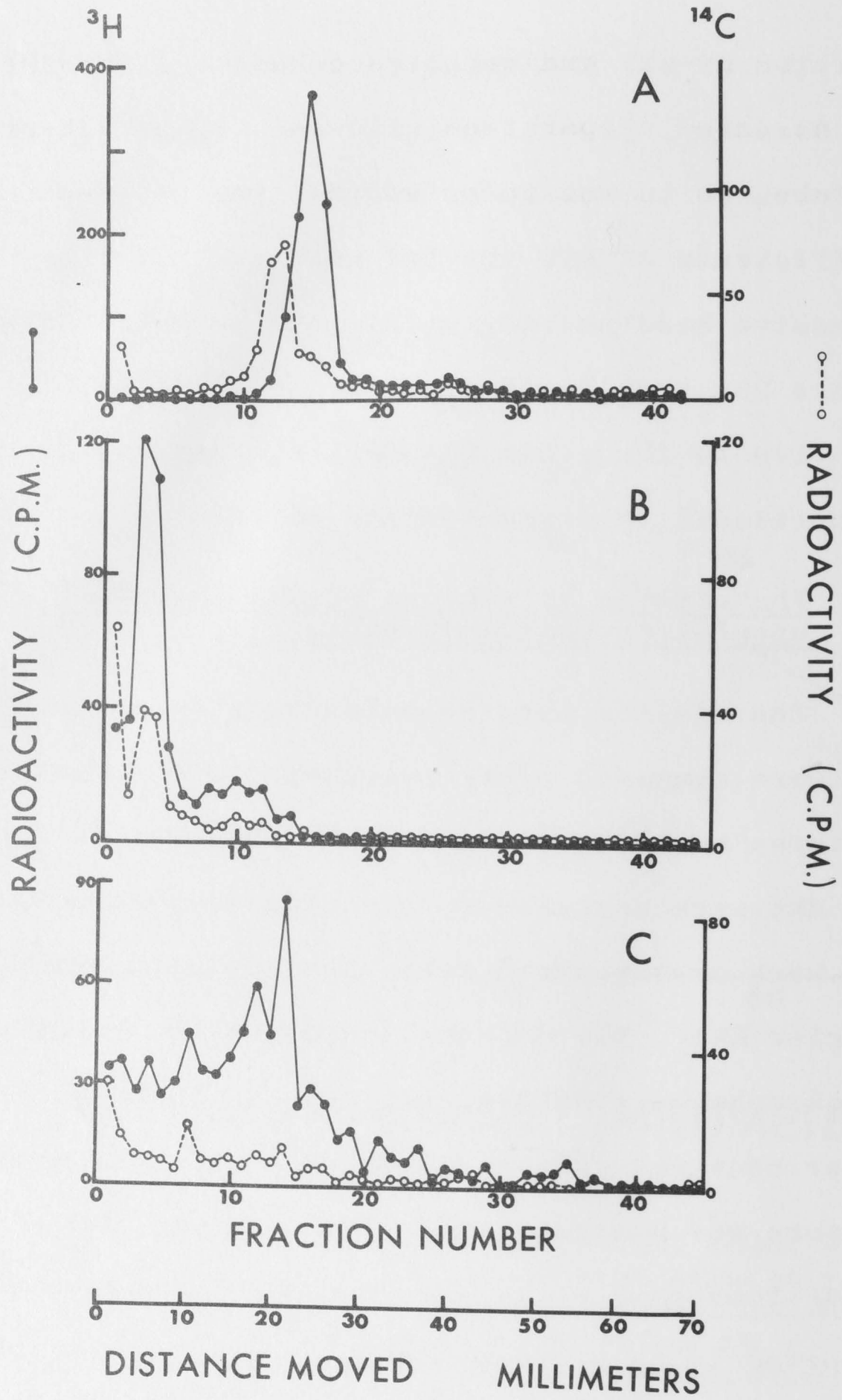


Figure 24: The influence of formaldehyde upon the electrophoretic mobilities of [^{3}H] TMV and [^{14}C[poliovirus RNA. A mixture of both RNA species was obtained as described in the text and was divided into 3 fractions (A,B and C). Fractions B and C were heated at 60^{o}C in the presence of 6 per cent formaldehyde and all mixtures were then submitted to electrophoresis at 70 volts and 6 m A per tube. Fraction A was electrophoresed for 4 hours, Fraction B for 3 hours and Fraction C for 6 hours and the distribution of radioactivity determined in the usual manner.

under the usual conditions (see Materials and Methods). The untreated preparation (A) was run for 4 hours at 7 mA per tube, while the formaldehyde-treated preparations (Band C) were run for 3 and 6 hours.

The results of the experiment, shown in Figure 24, indicate that, after formaldehyde treatment, an irregular type of electrophoretic migration occurs, with much of the input RNA remaining at the origin. Formaldehyde was not incorporated into the gels, as with the gradients because it prevented polymerisation, presumably by reacting with the acrylamide monomer. In its absence some refolding of the formaldehyde-treated RNA to assume original RNA structures may have occurred during migration. The reversibility of formaldehyde-RNA interaction has been noted by Fenwick (1968) after centrifuging formaldehyde-treated RNA through sucrose gradients containing no formaldehyde.

From the gradients in Figure 17, there is no evidence to suggest that scission of the RNA molecules of either poliovirus or TMV occurs as a result of heating at 60^{o} C in the presence of formaldehyde. Thus the conclusion from this experiment must be that secondary structures within these two RNA molecules are, partly at least, the determinants of relative electrophoretic mobility.

Summary

In comparative centrifugation studies with TMV RNA, the molecular weight of poliovirus RNA was estimated to be about 2.1 - 2.2. x 10^6 daltons. This figure was obtained after heating in the presence of, and centrifugation through sucrose gradients containing formaldehyde. It may, however, be an underestimate due to the relatively poor resolving power of such gradients. No difference between the sedimentation coefficients of poliovirus or TMV RNA could be detected in the presence of DMSO alone, but further studies revealed that formaldehyde, under the conditions of use, was probably a more effective RNA denaturant.

Poliovirus RNA had a higher molecular weight than that of bacteriophage R17 or SFV from similar comparative studies. The finding for SFV RNA was in contrast to some earlier estimates, derived from the sedimentation coefficients of undenatured viral RNA.

The molecular weight of poliovirus RNA when determined by its electrophoretic mobility in polyacrylamide gels was estimated to be 2.4 - 2.65 x 10^6 daltons. However, it was shown that RNA secondary structure could be a factor in this estimation.

DISCUSSION

1. Genetic Studies

As described in Experimental Section 1, additional ts mutants of poliovirus were obtained after treatment of infectious RNA with the exonucleasesnake venom phosphodiesterase or the mutagen nitrous acid. Snake venom phosphodiesterase removes individual nucleotides sequentially from the 3′- terminus of DNA or RNA polynucleotides (Razzell, 1963) and has been shown to cause a loss in poliovirus RNA infectivity (Holland et al., 1960). Infectious RNA was incubated with a low concentration of enzyme in attempts to produce terminal deletion mutants with ts defects specified by the gene adjacent to the 3′- terminus. In RNA bacteriophages a deletion of this kind would be expected to produce a ts defect in the gene specifying the viral RNA polymerase (August et. al., 1968; Spahr and Gesteland, 1968; Lodish, 1968), but whether the analogy is true for poliovirus is unknown.

Two mutants obtained after exposure of infectious RNA to snake venom phosphodiesterase, when examined by recombination analysis, were shown to possess ts defects near known defects of the RNA negative mutants ts-20 and -28.

However, the presence of revertants among these mutants and the likelihood of a specific binding site for RNA polymerase at the 3′- terminus from nucleotide sequence studies of several bacteriophage RNA templates (August, 1969), suggests that their occurrence is spontaneous, or at least brought about by some mutagenic function of venom phosphodiesterase unrelated to exonuclease activity. Little can be said of the specificity of the mutagen nitrous acid for different regions of the genome, as only a single mutant was examined in recombination tests.

The need to isolate mutants with specific markers at the extremities of the genome is apparent from a consideration of the coding potential of poliovirus RNA in relation to the genetic map in Figure 1. With a molecular weight in excess of 2.0×10^6 daltons (Experimental Section 3), poliovirus RNA has the capacity to translate proteins of total molecular weight at least 236,000 daltons. Virion proteins represented by ts defects in the right hand half of the map have a combined molecular weight of 92,500 daltons (Maizel and Summers, 1968). Other gene products, concerned with RNA synthesis but as yet uncharacterised, are represented by ts mutations in the left hand half.

This map may not carry representatives of considerable regions of the genome, such as those concerned with the inhibition during infection of cellular RNA and protein synthesis. Evidence that such inhibition is one of several coded functions of poliovirus RNA is suggested from observations that inhibition of macromolecular synthesis takes place in the presence of guanidine (which blocks viral RNA synthesis) (Bablanian et al., 1965) but is sensitive to puromycin (Penman and Summers, 1965). However, Martin and Kerr (1968) suggest that cellular proteins exert the inhibitory effect upon RNA and protein synthesis in response to infection. They argue that if the proteins responsible for inhibition were viral coded, then treatment of cells with interferon should prevent its expression. This has been shown not to be the case, suggesting that cellular proteins do have some role in inhibition.

If a viral-coded protein is involved, two possible explanations for failure to obtain mutants of this kind can be advanced:-

1. The size of the gene concerned may be small relative to other genes and this would affect the

isolation rate accordingly. It may, alternatively, be related to the base composition of the gene and the type of mutagen used, i.e., if the gene had a high purine content, then the probability of obtaining mutants with 5-FU would be low.

2. Another explanation (P.D. Cooper, personal communication) concerns the number of gene products required for the inhibition of macromolecular synthesis. Very few functional products of the inhibitor gene may be required for inhibition, and in mutants of the particular gene, sufficient copies of the gene product for inhibition of RNA and protein synthesis may be translated from the RNA of 'leak' or revertant progeny in the mutant population. Under these circumstances, such a defect would be very difficult to detect in tests of physiological function.

Cooper et al., obtained ts mutants of poliovirus that were defective in their capacity to inhibit cellular DNA synthesis during infection. If the same gene is required for inhibition of DNA synthesis as for the inhibition of RNA and protein synthesis, then a greater number of copies of the particular gene product may be required to bring about inhibition of DNA, and their

detection in a mutant population would thus be easier. Alternatively, two separate gene functions may be present, one concerned with DNA and the other with RNA and protein synthesis inhibition.

Evidence that only a small proportion of gene products is required for inhibition comes from the finding (Garwes, 1966) that the 'cell killing' property of certain ts mutants (measured by the estimate of cellular uptake of trypan blue) is not affected at 39.5°C, under conditions in which viral growth and the production of capsid antigen do not occur. It may be expected that fewer copies of all gene products are made in these circumstances, implying that a smaller number of molecules are required for this gene function than for, say, viral RNA synthesis.

In contrast to the situation for the hypothetical inhibitor protein, mutants with single defects in the genes which specify virion proteins are readily detectable in tests of physiological function, such as in vitro thermolability (McCahon and Cooper, 1969).

2. Polymerase Studies

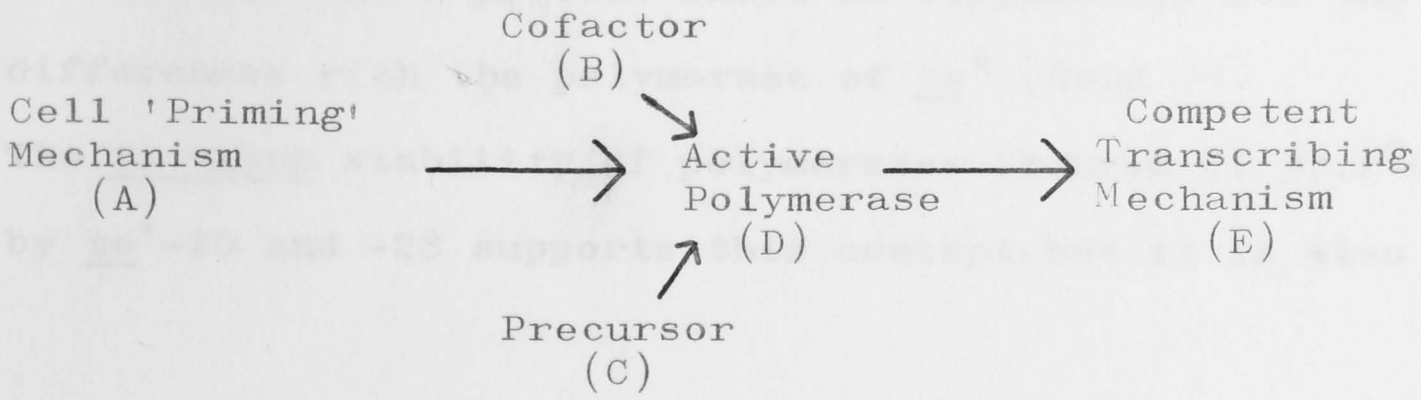
An enzyme activity was observed in cytoplasmic extracts of poliovirus-infected U cells with many properties of a viral RNA polymerase. This activity catalysed incorporation of ribonucleoside triphosphate into TCA insoluble material with the properties of RNA in the presence of the other three ribonucleoside triphosphates. It was shown that the product of the polymerase consisted of a complex of single- and double-stranded RNA and a similar product has been reported for the polymerases of mengovirus (Plagemann and Swim, 1968), foot-and-mouth disease virus (Arlinghaus and Polatnick, 1969) and poliovirus, prepared from infected HeLa cells (Girard, 1969). However, the highest sedimentation coefficient of the complex in Figure 10 was around 20S, compared with values of 28 S or greater for the complexes described by other workers, and there is evidence to suggest that this difference may be caused by the action of ribonucleases upon single-stranded RNA in the complex.

Preparations of polymerase induced by the RNA negative mutants ts-20 and -28 were shown to be quite stable in vitro compared with the polymerase of ts^{+} (Figures 15 and 16). However, mutant ts-20, in

comparison with ts$^+$ was defective in its capacity to induce polymerase during growth over a temperature range of 33° - 39.5°C (Figure 14). The optimum temperature for the induction of ts-20 polymerase was 34.5 - 35°C, compared with 37°C for ts$^+$

In vivo differences in the RNA produced in response to infection with ts-20 and -28 at 39.6°C indicate that two thermolabile gene functions are responsible for RNA synthesis (Cooper *et al.*, 1969). Mutant ts-20 was shown to be defective in its capacity to synthesise both single- and double-stranded RNA after growth at restrictive temperatures, whereas, under the same conditions, ts-28 allowed the synthesis of only double-stranded RNA. Cooper *et al.*, suggested that the ts defects of ts-20 and -28 were representative of enzymes I and II of the polymerase complex, being responsible for the synthesis of double- and single-stranded RNA, respectively.

The findings of Experimental Section 2 may be conveniently discussed according to the following scheme:-



Differences in polymerase levels induced by ts-20 compared with ts^+ (Figure 14) may be due to defects in a viral-coded cell 'priming' mechanism required for polymerase synthesis (A). However, 'late' defects in polymerase induction by ts^+ -20 and other mutants from step-up experiments (Figure 13) argue against such a mechanism, i.e., inhibition can occur even at times when considerable amounts of polymerase have been synthesised. These experiments do, however, suggest that the particular gene product concerned has a rapid turnover.

These observed differences may be due not to the induction of low polymerase levels by ts-20, but to mistakes in the transcribing mechanism of substituted polymerases during *in vitro* incubation (E). However, evidence that this is not so can be seen in Figure 16, where no difference in the activity of ts-20 polymerase compared with that of ts^+ and -28 occurred over a range of temperatures.

A substituted cofactor or precursor induced by ts-20 and not the actual polymerase molecule may be the thermolabile gene product which is responsible for the differences with the polymerase of ts^+ (Band C). The *in vitro* stability of polymerases induced at 37.2°C by ts^+-20 and -28 supports this concept, but it is also

possible that ts-20 polymerase is unstable *in vivo*, compared with ts^+, but becomes stabilised during extraction from cells. Given the data in Experimental Section 2, it is not possible to distinguish between either possibility.

Martin (1969) was also unable to detect differences in the *in vitro* stability of polymerases induced by RNA negative mutants of Semliki Forest virus and by the wild type strain. Priess and Eggers (1968) found that a thermosensitive event in the production of viral polymerase was associated with the inability of a Sabin vaccine strain of poliovirus to replicate at temperatures around 40°C. Once induced, the polymerase was stable at 40°C. However, the nature of the genetic defect or defects in Sabin strains is completely unknown (they are probably multiple; P.D. Cooper, personal communication), and it is not possible at present to draw comparisons between the polymerases of Sabin strains and RNA negative mutants.

3. Molecular Weight Studies with Viral RNA

Poliovirus RNA was shown to have a molecular weight greater than the figure of 2.0×10^6 daltons accepted for TMV RNA (Boedtker, 1960), but the precise value for poliovirus RNA cannot yet be determined due to uncertainties in the interpretation of results obtained by different methods. Uncertainties arise from:-

1. Lack of information as to whether formaldehyde or DMSO fully eliminate RNA secondary structure in centrifugation studies i.e., whether any additional bonding exists between groups that do not react with these denaturants.

2. Doubts as to the capacity of 15-30 per cent (W/V) sucrose gradients containing formaldehyde fully to resolve differences in molecular weight between poliovirus and TMV RNA that are suggested from sedimentation studies.

3. The possibility that RNA secondary structure as well as molecular weight may be a determinant of electrophoretic mobility in polyacrylamide gels.

There is much evidence to suggest that all RNA secondary structures are removed after treatment with formaldehyde at 60°C or DMSO at room temperature (Boedtker, 1967, 1968a; Katz and Penman, 1966; Strauss

et al., 1968). However, when mixtures of TMV and '28 S' ribosomal RNA were treated in gradients containing either denaturant, a higher molecular weight for '28 S' ribosomal RNA over TMV RNA was obtained in the presence of formaldehyde. This result may be peculiar to the use of '28 S' ribosomal RNA with unusual intramolecular structures, as was suggested by Fenwick (1968), but it also raises some doubts as to the efficacy of both formaldehyde and (particularly) DMSO as denaturants.

If formaldehyde is a complete denaturant for the types of RNA examined in Experimental Section 3, then the resolution afforded in 15-30 per cent sucrose gradient containing formaldehyde is relatively poor. This is suggested from Figure 19 where the peak for poliovirus RNA, with an anticipated twofold difference in molecular weight over R17 RNA, appears only two fractions ahead of the latter. Under these circumstances, relatively larger differences may exist in molecular weight between poliovirus and TMV RNA than are suggested from Figure 17 C and D.

A good correlation between electrophoretic mobility and molecular weight was obtained for several RNA species (Figures 22, 23) which normally have varying degrees of

internal secondary structure (Gesteland and Boedtker, 1964; Sprecher-Goldberger, 1967). This gave a molecular weight for poliovirus RNA of 2.40 - 2.65 x 10^6 daltons by extrapolation. However, when RNA secondary structures in poliovirus and TMV RNA were removed with formaldehyde prior to electrophoresis, a pattern unrelated to molecular weight was obtained (Figure 24). This may be due to partial refolding of the RNA species in the absence of formaldehyde or to binding of the RNA to the gel.

It would be desirable in electrophoretic studies with poliovirus RNA to use RNA markers with molecular weights in the range 2.0 - 3.0 x 10^6 daltons, in order to avoid the need for extrapolation. Two recently characterised RNA species, 32 S ribosomal precursor RNA (M.W. 2.4 x 10^6; McConkey and Hopkins, 1969) and Rous sarcoma virus RNA (M.W. approximately 3.0 x 10^6; Duesberg 1968b) might be useful in this regard.

ACKNOWLEDGEMENTS

I am indebted to my supervisor Dr P.D. Cooper for much helpful advice and consideration during the course of this work and in the writing of the thesis. I am also grateful to Professors G.L. Ada and F.J. Fenner for the privilege of working in their department.

Thanks are also due to various colleagues within the Department of Microbiology for advice and assistance, notably Drs D. McCahon, E.M. Martin, A.J. Gibbs, C.J. Burrell and A.J.D. Bellett and to my wife Jessica for much forebearance over the past three years. Mrs A. Kemenes assisted with the preparation of Figures.

I was supported during my tenure at the Australian National University by a Commonwealth Public Service Post-Graduate Scholarship, while on leave of absence from the National Biological Standards Laboratory. I thank the Commonwealth Public Service Board for granting me this award.

BIBLIOGRAPHY

Ackermann, W.W., Loh, P.C., and Payne, F.E. (1959). Virology 7, 170.

Arlinghaus, R.B., and Polatnick, J. (1969). Proc. Natl. Acad. Sci., U.S., 62, 821.

Anderer, F.A., and Restle, H. (1964). Z. Naturforsch, 19b, 1026.

August, J.T. (1969). Nature 222, 121.

August, J.T., Banerjee, A.K., Eoyang, L., Franze de Ferdinandez, M.T., Hasegawa, S., Hori, K., Kuo, C.H., Rensing, U., and Shapiro, L. (1968). Cold Spring Harb. Symp. Quant. Biol., 33, 73.

Bablanian, R., Eggers, H.J., and Tamm, I. (1965). Virology 26, 100.

Baltimore, D. (1964). Proc. Natl. Acad. Sci., U.S., 51, 450.

Baltimore, D. (1966). J. Mol. Biol., 18, 421.

Baltimore, D. (1968). J. Mol. Biol., 32, 359.

Baltimore, D., and Franklin, R.M. (1963). J. Biol. Chem., 238, 3395.

Baltimore, D., and Girard, M. (1966). Proc. Natl. Acad. Sci., U.S., 56, 741.

Baltimore, D. and Huang, A.S. (1968). Quoted in Jacobson and Baltimore (1968a).

Baltimore, D., Becker, Y., and Darnell, J.E., Jr (1964). Science (N.Y.), 143, 1034.

Baltimore, D., Girard, M., and Darnell, J.E., Jr (1966). Virology 29, 179.

Baltimore, D., Eggers, H.J., Franklin, R.M., and Tamm I. (1963). Proc. Natl. Acad. Sci., U.S., 49, 843.

Bellamy, A.R., and Joklik, W.K. (1967). J. Mol. Biol., 21, 19.

Bishop, J.M., and Koch, G. (1967). J. Biol. Chem., 242, 1736.

Bishop, J.M., and Koch, G. (1969). Virology, 37, 521.

Bishop, D.H.L., Claybrook, J.R., and Spiegelman, S. (1967). J. Mol. Biol., 26, 373.

Bishop, J.M., Summers, D.F., and Levintow, L. (1965). Proc. Natl. Acad. Sci., U.S., 54, 1273.

Boedtker, H. (1960). J. Mol. Biol. 2, 171.

Boedtker, H. (1967). Biochemistry 6, 2718.

Boedtker, H. (1968a). J. Mol. Biol. 35, 61.

Boedtker, H. (1968b). In Methods in Enzymology, vol.XII Pt.B, (eds. L. Grossman and K. Moldave), p.429.

Boeyé, A. (1959). Virology 9, 691.

Boeyé, A. (1962). Nature 193, 601.

Bratt, M.A., and Robinson, W.S. (1967). J. Mol. Biol. 23, 1.

Brenner, S., Stretton, A.O.W., and Kaplan, S. (1965). Nature 206, 994.

Brown, F., and Martin, S.J. (1965). Nature 208, 861.

Brownhill, T.J., Jones, A.S., and Stacey, M. (1959). Biochem. J. 73, 434.

Burge, B.W., and Pfefferkorn, E.R. (1964). Virology 24, 126.

Burrell, C.J., Martin, E.M. and Cooper, P.D. (1969). In preparation.

Campbell, A. (1961). Virology 14, 22.

Campbell, A., and del Campillo-Campbell, A. (1963). J. Bact. 85, 1202.

Carp, R.I., and Koprowski, H. (1962). Virology 17, 99.

Chantrenne, H., Burny, A., and Marbaix, G. (1967). Prog. Nuc. Acid Res. Mol. Biol. 7, 173.

Conteras, G., Tohá, J. and Ohlbaum, A. (1959). Biochim. Biophys. Acta 35, 268.

Cooper, P.D. (1959). Virology 7, 469.

Cooper, P.D. (1961). Virology 13, 153.

Cooper, P.D. (1964a). J. Gen. Microbiol. 37, 2.

Cooper, P.D. (1964b). J. Gen. Microbiol. 37, 267.

Cooper, P.D. (1964c). Virology 22, 186.

Cooper, P.D. (1965). Virology 25, 431.

Cooper, P.D. (1966). Virology 28, 663.

Cooper, P.D. (1968). Virology 35, 584.

Cooper, P.D. (1969). In The Biochemistry of Viruses (ed. H.B. Levy). Marcel Dekker inc., N.Y., 177.

Cooper, P.D., Johnson, R.T., and Garwes, D.J. (1966). Virology 30, 638.

Cooper, P.D., Summers, D.F. and Stancék, D. (1969). In preparation.

Cooper, P.D., Wentworth, B.B. and McCahon, D. (1969). In preparation.

Cords, C.E. and Holland, J.J. (1964). Virology 24, 492.

Crocker, T.T., Pfendt, E., and Spendlove, R. (1964). Science (N.Y.), 145, 401.

Dales, S. and Kajioka, R. (1964). Virology 24, 278.

Dalgarno, L. and Martin, E.M. (1965). Virology 26, 450.

Dalgarno, L., Martin, E.M., Liu, S.-L. and Work, T.S. (1966). J. Mol. Biol. 15, 77.

Darnell, J.E., Jr (1962). Cold Spring Harb. Symp. Quant. Biol. 27, 149.

Darnell, J.E., Jr, and Sawyer, T.K. (1960). Virology 11, 665.

Darnell, J.E., Jr, Levintow, L., Thoren, M.M., and Hooper, J.L. (1961). Virology 13, 271.

Davis, B.J. (1964). Ann. N.Y. Acad. Sci. 121, 404.

Denhardt, D.T., and Sinsheimer, R.L. (1965). J. Mol. Biol. 12, 647.

de Waard, A., Paul, A.V., and Lehman, I.R. (1965). Proc. Natl. Acad. Sci. U.S. 54, 1241.

Diener, T.O., and Schneider, I.R. (1968). Arch. Biochem. Biophys. 124, 401 (1968).

Dixon, M., and Webb, E.C. (1958). Enzymes, Longman, Green and Co., London, N.Y., Toronto, p.62.

Doty, P., and Edsall, J.T. (1951). Advan. Protein Chem. 6, 35.

Duesberg, P.H. (1968a). Proc. Natl. Acad. Sci. U.S. 59, 930.

Duesberg, P.H. (1968b). Proc. Natl. Acad. Sci. U.S. 60, 1511.

Dulbecco, R. (1952). Proc. Natl. Acad. Sci. U.S. 38, 747.

Dulbecco, R. (1961). In Poliomyelitis: papers and discussion, 5th international poliomyelitis conference, Copenhagen, Lippincott, Pa., p.21.

Dulbecco, R., and Vogt, M. (1954). J. Exp. Med. 99, 167.

Eagle, H. (1959). Science (N.Y.) 130, 432.

Edgar, R.S., and Wood, W.B. (1966). Proc. Natl. Acad. Sci. U.S. 55, 498.

Eggers, H.J., Baltimore, D., and Tamm, I. (1963). Virology 21, 281.

Eggers, H.J., Reich, E., and Tamm, I. (1963). Proc. Natl. Acad. Sci. U.S. 50, 183.

Eikhom, T.S., Stockley, D.J., and Spiegelman, S. (1968). Proc. Natl. Acad. Sci., U.S., 59, 506.

Epstein, R.H., Bollé, A., Steinberg, C.M., Kellenberger, E., Boy de la Tour, E., Chevalley, R., Edgar, R.S., Susman, M., Denhardt, G.H., Leilausis, A. (1963). Cold Spring Harb. Symp. Quant. Biol. 28, 375.

Erikson, R.L., and Franklin, R.M. (1966). Bact. Rev. 30, 267.

Erikson, R.L., Fenwick, M.L., and Franklin, R.M. (1965). J. Mol. Biol. 13, 399.

Fazekas de St. Groth, S. (1948). Nature 162, 294.

Feix, G., Pollet, R., and Weissman (1968). Proc. Natl. Acad. Sci. U.S. 59, 145.

Feix, G., Slor, H., and Weissman, C. (1967). Proc. Natl. Acad. Sci. U.S. 57, 1401.

Fenner, F. (1968). The Biology of Animal Viruses, Academic Press, N.Y. and Lond., p.100.

Fenwick, M.L. (1963). Virology 19, 241.

Fenwick, M.L. (1968). Biochem. J. 107, 851.

Fenwick, M.L., and Cooper, P.D. (1962). Virology 18, 212.

Fields, B.N., and Joklik, W.K. (1969). Virology 37, 335.

Fraenkel-Conrat, H., Singer, G., and Tsugita, A. (1961). Virology 14, 54.

Franklin, R.M., and Baltimore, D. (1962). Cold Spring Harb. Symp. Quart. Biol. 27, 175.

Franze de Ferdinandez, M.T., Eoyang, L., and August, J.T. (1968). Nature 219, 588.

Fried, M. (1965). Virology 25, 669.

Friedman, R.M., Levy, H.R., and Carter, W.B. (1966). Proc. Natl. Acad. Sci. U.S. 56, 440.

Garwes, D.J. (1966). Cellular Changes induced by Poliovirus. Ph.D. thesis Australian National University, Canberra.

Gesteland, R.F., and Boedtker, H. (1964). J. Mol. Biol. 8, 496.

Gierer, A. (1957). Nature 179, 1297.

Gierer, A. (1958). Z. Naturforsch. 136, 477.

Gilliland, J.M., and Symons, R.H. (1968). Virology 36, 232.

Ginoza, W. (1958). Nature, 181, 958.

Girard, M. (1969). J. Virol. 4, 376.

Girard, M., Baltimore, D., and Darnell, J.E., Jr (1967). J. Mol. Biol. 24, 59.

Grado, C., Fischer, S., and Conteras, G. (1965). Virology 27, 623.

Gussin, G.N. (1966). J. Mol. Biol. 21, 435.

Hamilton, M.G. (1967). Biochim. Biophys. Acta 134, 475.

Hanks, J.H., and Wallace, R.E. (1949). Proc. Soc. Exp. Biol. Med. 71, 196.

Haselkorn, R., and Doty, P. (1961). J. Biol. Chem. 236, 2738.

Hayes, W. (1964). The Genetics of Bacteria and Their Viruses. Blackwell, Oxford, pp.228, 280.

Hershey, A.D. (1955). Virology 1, 108.

Hirst, G.K. (1962). Cold Spring Harb. Symp. Quart. Biol. 27, 303.

Holland, J.J. (1962). Biochem. Biophys. Res. Comm. 9, 556.

Holland, J.J. (1963). Proc. Natl. Acad. Sci. U.S. 49, 23.

Holland, J.J., and Bassett, D.W. (1964). Virology 23, 164.

Holland, J.J., and Hoyer, B.H. (1962). Cold Spring Harb. Symp. Quart. Biol. 27, 101.

Holland, J.J., and Kiehn, D. (1968). Proc. Nat. Acad. Sci. U.S. 60, 105.

Holland, J.J., and McClaren, L.C. (1961). J. Exp. Med. 114, 161.

Holland, J.J., and Peterson, J.A. (1964). J. Mol. Biol. 8, 556.

Holland, J.J., McLaren, L.C., Hoyer, B.H., and Syverton, J.T. (1960). J. Exp. Med. 112, 841.

Horton, E., Liu, S.-L., Martin, E.M., and Work, T.S. (1966). J. Mol. Biol. 15, 62.

Horton, E., Liu, S.-L., Dalgarno, L., Martin, E.M., and Work, T.S. (1964). Nature 204, 247.

Hotham-Iglewski, B., and Franklin, R.M. (1967). Proc. Natl. Acad. Sci. U.S. 58, 743.

Howes, D.W. (1959). Virology 9, 110.

Howes, D.W., and Melnick, J.L. (1957). Virology 4, 97.

Jacobson, M.F., and Baltimore, D. (1968a). Proc. Natl. Acad. Sci. U.S. 61, 77.

Jacobson, M.F., and Baltimore, D. (1968b). J. Mol. Biol. 33, 369.

Joklik, W.K., and Darnell, J.E., Jr (1961). Virology 13, 439.

Kamen, R. (1969). Nature, 221, 321.

Katz, L., and Penman, S. (1966). Biochem. Biophys. Res. Comm. 23, 557.

Kelly, R.B., and Sinsheimer, R.L. (1967). J. Mol. Biol. 29 237.

Kiho, Y., and Rich, A. (1964). Proc. Natl. Acad. Sci. U.S. (1964) 51, 111.

Kirvaitis, J., and Simon, E.H. (1965). Virology 26, 545.

Koch, G., Quintrell, N., and Bishop, J.M. (1966). Biochem. Biophys. Res. Comm. 24, 304.

Kretsinger, R.H., Manner, G., Gould, B.S., and Rich, A. (1964). Nature 202, 438.

Kuff, E.L., and Roberts, N.E. (1967). J. Mol. Biol. 26, 211.

Kurland, C.G. (1960). J. Mol. Biol. 2, 83.

Ledinko, N. (1963). Virology 20, 107.

Levintow, L., and Darnell, J.E., Jr (1960). J. Biol. Chem. 235, 70.

Levintow, L., Thoren, M.M., Darnell, J.E., Jr, and Hooper, J.L. (1962). Virology 16, 220.

Levy, H.B. (1961). Virology 15, 173.

Lodish, H.F. (1968). J. Mol. Biol. 32, 681.

Lodish, H.F., and Zinder, N.D. (1966). Science (N.Y.) 152, 372.

Loeb, T. and Zinder, N.D. (1961). Proc. Natl. Acad. Sci. U.S. 47, 282.

Loening, U.E. (1967). Biochem. J. (1967) 102, 251.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). J. Biol. Chem. 193, 165.

Lwoff, A. and Lwoff, M. (1959a). C.r. hebd. Séanc. Acad. Sci., Paris 248, 154.

Lwoff, A. and Lwoff, M. (1959b). C.r. hebd. Séanc. Acad. Sci., Paris 248, 1725.

Maasab, H.F., Loh, P.C., and Ackermann, W.W. (1957). J. Exp. Med. 106, 641.

Maizel, J.V., Jr and Summers, D.F. (1968). Virology 36, 48.

Mandel, B. (1967). Virology 31, 702.

Marcus, P.I., and Freiman, M.E. (1966). Methods Cell Physiol. 2, 93.

Martin, E.M. (1967). Mechanisms of Viral RNA Replication. In Genetic Elements, Properties and Functions. (ed. D. Shugar), Academic Press, N.Y., p.117.

Martin, E.M. (1969). Virology 39, 107.

Martin, E.M., and Kerr, I.M. (1968). Symp. Soc. Gen. Microbiol. 18, 15.

Martin, E.M., and Sonnabend, J.A. (1967). J. Virol. 1, 97.

Mills, D.R., Pace, N.R. and Spiegelman, S. (1966). Proc. Natl. Acad. Sci. U.S. 56, 1778.

Montagnier, L., and Sanders, F.K. (1963). Nature 199, 664.

Morse, D.E., Baker, R.F., and Yanofsky, C. (1968). Proc. Natl. Acad. Sci. U.S. 60, 1428.

Munyon, W. and Salzman, N.P. (1962). Virology 18, 95.

McBride, W.D. (1962). Virology 18, 118.

McCahon, D. (1967). Studies on temperature-sensitive poliovirus mutants. Ph.D. thesis, Australian National University, Canberra.

McCahon, D., and Cooper, P.D. (1969). In preparation.

McConkey, E.H., and Hopkins, J.W. (1969). J. Mol. Biol. 39, 549.

MacKenzie, J.S. (1968). Genetic studies with influenza virus. Ph.D. thesis, Australian National University, Canberra.

MacPherson, I. and Stoker, M. (1962). Virology 16, 147.

Nathans, D., Oeschger, M.P., Eggen, K., Shimura, Y. (1966). Proc. Natl. Acad. Sci. U.S. 56, 1844.

Nayak, D.P., and Baluda, M.A. (1968). Proc. Natl. Acad. Sci. U.S. 59, 185.

Ohtaka, Y. and Spiegelman, S. (1963). Science (N.Y.). 142, 493.

Penman, S., and Summers, D.F. (1965). Virology 27, 614.

Penman, S., Becker, Y., and Darnell, J.E., Jr (1964). J. Mol. Biol. 8, 541.

Penman, S., Scherrer, K., Becker, Y., and Darnell, J.E., Jr (1964). Proc. Natl. Acad. Sci. U.S. 49, 654.

Petermann, M.L., and Pavlovec, A. (1966). Biochim. Biophys. Acta 114, 264.

Philipson, L. (1963). Prog. Med. Virol. 5, 43.

Pittman, D. (1965). Genetics, Princeton 52, 468.

Plagemann, P.G.W., and Swim, H.E. (1966). Bact. Rev. 30, 288.

Plagemann, P.G.W., and Swim, H.E. (1968). J. Mol. Biol. 35, 13.

Pohjanpelto, P. (1961). Virology 15, 225.

Pohjanpelto, P., and Cooper, P.D. (1965). Virology 25, 350.

Polatnick, J., and Arlinghaus, R.B. (1967). Virology 31, 601.

Pons, M. (1964). Virology 24, 467.

Priess, H., and Eggers, H.J. (1968). Nature 220, 1047.

Razzell, W.E. (1963). In Methods in Enzymology (eds. S. Colowick, and N.O. Kaplan) Vol.VI, 236. Academic Press, N.Y. and London.

Razzell, W.E. and Khorana, H.G. (1959). J. Biol. Chem. 234, 2114.

Rayleigh, Lord (1871). Phil. Mag. 41, 107.

Reich, E., Franklin, R.M., Shatkin, A.J., and Tatum, E.L. (1961). Science (N.Y.) 134, 556.

Salzman, N.P., Lockart, R.Z., Jr and Sebring, E.D. (1959). Virology 9, 244.

Sambrook, J.R., Padgett, B.L., and Tomkins, J.K.N. (1966). Virology 28, 592.

Schaffer, F.L. (1962). Cold Spring Harb. Symp. Quant. Biol. 27, 89.

Schaffer, F.L., and Frommhagen, L.H. (1965). Virology 25, 662.

Schaffer, F.L., and Gordon, M. (1966). J. Bact. 91, 2309.

Scharaga, H.A., and Mandelkern, L. (1953). J. Amer. Chem. Soc. 75, 179.

Scharff, M.D., Shatkin, A.J., and Levintow, L. (1963). Proc. Natl. Acad. Sci. U.S. 50, 686.

Shapiro, A.L., Scharff, M.D., Maizel, J.V., Jr and Uhr, J.W. (1966). Proc. Natl. Acad. Sci. U.S. 56, 216.

Scholtissek, C., and Rott, R. (1969). J. Gen. Virol. 4, 125.

Schuster, H., and Schramm, G. (1958). Z. Naturforsch 13b, 697.

Sever, J.L. (1962). J. Immunol. 88, 320.

Silverstein, S.C., and Marcus, P.I. (1964). Virology 23, 370.

Sonnabend, J.A., Martin, E.M., and Mécs, E. (1967). Nature 213, 365.

Spahr, P.F. and Gesteland, R.F. (1968). Proc. Natl. Acad. Sci. U.S. 59, 876.

Spiegelman, S., Haruna, I., Holland, I.B., Beaudreau, G., and Mills, D. (1965). Proc. Natl. Acad. Sci. U.S. 54, 919.

Spirin, A.S. (1961). Biokhimiya 26, 511 (Eng. trans. p.454).

Sprecher-Goldberger, S. (1967). Arch. ges. Virusforsch. 20, 225.

Sreevalsan, T., Lockart, R.Z. Jr, Dodson, M.L. Jr, and Hartman, K.A. J. Virol. 2, 558.

Stanley, W.M. Jr, and Bock, R.M. (1965). Biochem. 4, 1302.

Strauss, J.H., and Sinsheimer, R.L. (1963). J. Mol. Biol. 7, 43.

Strauss, J.H., Kelly, R.B., and Sinsheimer, R.L. (1968). Biopolymers 6, 793.

Stretton, A.O.W., and Brenner, S. (1965). J. Mol. Biol. 12, 456.

Summers, D.F., and Levintow, L. (1965). Virology 27, 44.

Summers, D.F., and Maizel, J.V., Jr (1968). Proc. Natl. Acad. Sci. U.S. 59, 966.

Summers, D.F., Maizel, J.V. Jr (1965). Proc. Natl. Acad. Sci. U.S. 54, 505.

Takemoto, K.K., and Leibhaber, H. (1962). Virology 17, 499.

Tan, K.B., Sambrook, J.F., and Bellett, A.J.D. (1969). Virology 38, 427.

Tershak, D.R. (1966). J. Mol. Biol. 21, 43.

Tooze, J., and Weber, K. (1967). J. Mol. Biol. 28, 311.

Viñuela, E., Algranati, I.D., Ochoa, S. (1967). Eur. J. Biochem. 1, 3.

Vogt, M., Dulbecco, R., and Wenner, H.A. (1957). Virology 4, 141.

Warner, J., Madden, M.J., and Darnell, J.E., Jr. (1963). Virology 19, 393.

Warner, J.R., Knopf, P., and Rich, A. (1963). Proc. Natl. Acad. Sci. U.S. 49, 122.

Watanabe, M., and August, J.T. (1967). In Methods in Virology. Vol.III (eds. K. Maramorosch and H. Koprowski), p.337. Academic Press, N.Y. and London.

Wecker, E. and Schonne, E. (1961). Proc. Natl. Acad. Sci. U.S. 47, 278.

Weissmann, C., Simon, L., and Ochoa, S. (1963). Proc. Natl. Acad. Sci. U.S. 49, 407.

Weissmann, C., Borst, P., Burdon, R.H., and Ochoa, S. (1964). Proc. Natl. Acad. Sci. U.S. 51, 890.

Wentworth, B.B., McCahon, D., and Cooper, P.D. (1968). J. Gen. Virol. 2, 297.

Willems, M. and Penman, S. (1966). Virology 30, 355.

Williamson, A.R., and Askonas, B.A. (1967). J. Mol. Biol. 23, 201.

Wilson, J.N., and Cooper, P.D. (1962). Virology 17, 195

Zimm, B.H. (1968). J. Chem. Phys. 16, 1093.

Zimmerman, E.F., Heeter, M. and Darnell, J.E., Jr (1963). Virology 19, 400.

Zinder, N.D. (1965). A. Rev. Microbiol. 19, 455.